PRODUCTION, CHARACTERIZATION AND USES OF A FUNGAL BIOPOLYMER ISOLATED FROM DIFFERENT SOURCES – A COMPARISON

Dissertation submitted to

The Tamil Nadu Dr. M.G.R. Medical University, Chennai

in partial fulfilment of the award of degree of

MASTER OF PHARMACY

(PHARMACEUTICAL BIOTECHNOLOGY)

Submitted by

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Under the guidance of

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Head, Dept. of Pharmaceutical Biotechnology

MARCH – 2008

COLLEGE OF PHARMACY

SRI RAMAKRISHNA INSTITUTE OF PARAMEDICAL SCIENCES

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in the Department of Pharmaceutical Biotechnology, College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences, Coimbatore, which is affiliated to The Tamilnadu Dr. M.G.R. Medical University, Chennai, under my direct supervision and co-guidance to my fullest satisfaction.

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Bharathi. P

I. SCOPE AND PLAN OF WORK

The aim of the present work was to isolate and characterize chitosan from fungi and explore some of its applications. This work was taken up for the following purposes.

1. To produce a biopolymer.
2. To overcome the disadvantages of the traditional processes of chitosan production using crustacean shells.

1. To produce a biopolymer

Worldwide every year approximately 140 million tonnes of synthetic polymers are produced. Since these polymers are stable, their degradation cycle in the biosphere is limited. This necessitates the need for the natural biodegradable polymers from the renewable sources which fit into the ecological cycle. Nowadays, a variety of biopolymers have become available for use in many applications that are biocompatible and eco-friendly. Among this chitin and its derivative chitosan have been chosen for this study because of their versatile applications in pharmaceutical, biomedical, food and agricultural fields.

2. To overcome the disadvantages of the traditional processes of chitosan production using crustacean shells.

Currently, commercial chitosan is mostly produced from the crustacean shells. This source contains a high percentage of $CaCO_3$ which releases CO_2 into the environment which cannot be considered to be an eco-friendly process. The seasonal supply of crustacean shells and lengthy extraction process contributes to high production costs that limit the actual industrial demand of this polymer.

Perhaps, the most sustainable alternative source in the long run is fungal mycelial wastes as it contains chitin and chitosan in their cell wall components. The non-animal origin of the fungal chitosan avoids the risk of allergy, reduces the extraction time and cost by growing on simple medium. It also controls the molecular weight and degree of deacetylation of chitosan.

In the current study, *Aspergillus Niger* NCIM 545, *Rhizopus oryzae* NCIM 879 and *Mucor hiemalis* NCIM 873 were used as test organisms for the production of biopolymer, **Chitosan**.

We intended to isolate, optimize, characterize, identify and study the uses of the chitosan from *Aspergillus niger* NCIM 545*, Rhizopus oryzae* NCIM 879 and *Mucor hiemalis* NCIM 873.

Thus the research was focused to attain the following goals.

1. To screen newer chitosan producing fungal strains.
2. To evaluate the optimal harvesting time of mycelia for maximal chitosan production.
3. To find out the suitable carbon and nitrogen source for the maximal production of chitosan.
4. To characterize isolated chitosan by FT-IR spectroscopy, NMR spectroscopy and viscometry.
5. To evaluate fungal chitosan for its anti-oxidant and anti-microbial activities.

ABSTRACT OF THE WORK

Chitin is highly insoluble N - acetylated polymer of β- (1, 4)-D-glucosamine. Chitosan is an acid soluble deacetylated form of chitin. Chitin is commonly found in exoskeleton of marine invertebrates and cuticles of insects. Both chitin and chitosan are also present in the cell wall of most fungi. Chitosan can be obtained by deacetylating chitin prepared from crab or shrimp shell. However, this process fails to produce chitosan of uniform quality. The physicochemical characteristics of chitosan depend on the degree of deacetylation and it also differs, based on the crustacean species and preparation methods used. Chitosan can also be obtained from filamentous fungi. No chemical deacetylation is necessary in this process. As a result, the quality of fungal chitosan is more consistent, inexpensive, eco friendly and chitin and chitosan are extracted simultaneously. Therefore the physicochemical properties and yield of chitosan isolated directly from fungi may be optimized by controlled fermentation. In this work fungal chitosan was prepared from *Aspergillus niger, Rhizopus oryzae* and *Mucor hiemalis* without using strong alkaline solution normally used for deacetylation. Fungal chitosan was isolated, with 85-95% degree of deacetylation and viscosity of 13-18 (cP). The profile of both isolated and commercial chitosan showed similar FT-IR and ^{1}HNMR spectra. The antioxidant activity of the isolated chitosan was studied using the stable DPPH (2, 2 diphenyl- picryl- hydrazyl-hydrate) free radical method. Ascorbic acid was used as standard. The fungal chitosan showed maximum scavenging of DPPH free radicals (EC_{50} value was in the range of 3.1- 5.3). The results suggest that chitosan possesses significant *in vitro* antioxidant activity compared with ascorbic acid. The antibacterial activity of the isolated chitosan was studied by Kirby-Bauer method and it showed good activity against *S. aureus*, *E.coli,* including clinical isolate of *E.coli*.

III. INTRODUCTION

3.1Polymers

Approximately 140 million tonnes of synthetic polymers are produced worldwide every year. Since polymers are extremely stable, their degradation cycles in the biosphere unlimited. Environmental pollution by synthetic polymers, such as waste plastics and water soluble synthetic polymers in waste water has been recognized as a major problem. Plastics and polymers are an integrated part of our daily existence. However, because of stability and resistance to degradation these are accumulated in the environment, at the rate of about 8% by weight and 20% by volume of the landfills. (Premraj and Doble 2005)

3.1.1Biopolymers

Biodegradable polymers are growing in importance, by the day and current research is focused on producing newer biodegradable polymer. A vast number of biodegradable polymers have been synthesized or are formed in nature during the growth cycles of all organisms. Some microorganisms and enzymes capable of degrading them have been identified. Eg: Depending to the evolution of the synthesis, Different classifications of the biodegradable polymers have been proposed. The classification based on biosynthetic process includes four different categories of which the first three categories are obtained from renewable resources

1. Polymers from biomass such as the agro-polymers from agro-resources (e.g., starch, cellulose, proteins like casein and gluten)
2. Polymers obtained by microbial production, e.g., Polyhydroxy-alkanoates.
3. Polymers conventionally and chemically synthesized and whose the monomers are obtained from agro-resources, e.g., the poly(lactic acid)
4. Polymers whose monomers and polymers are obtained conventionally, by chemical synthesis.(Petersen K., et al., 1999)

3.3. Chemical classification of biopolymers

Organisms are able to synthesize an overwhelming variety of polymers which can be distinguished into eight major classes according to their chemical structure.

1. Nucleic acids.
2. Polyamides such as proteins and poly amino acids.
3. Polysaccharides.
4. Organic polyesters such as poly (hydroxyalkanoic acids), poly (malic acid) and cutin.
5. Polythioesters, (which were only reported recently).
6. Inorganic polyesters with polyphosphate.
7. Polyisoprenoides such as natural rubber or Gutta Percha.
8. Polyphenols such as lignin or humic acids.

3.4. Functions and synthesis of biopolymers

Living matter is able to synthesize a wide range of different polymers, and in most organisms these biopolymers contribute the major fraction of cellular dry matter. The functions of biopolymers are in most cases, essential for the cells and are as manifold as their structures. These biopolymers fulfill a range of quite different essential functions for the organisms such as

- Conservation and expression of genetic information.
- Catalysis of reactions, storage of carbon, energy or other nutrients.
- Defending and protecting against the attack of other cells, hazardous environmental factors, sensing of biotic and abiotic factors.
- Communication with the environment and other organisms.
- Mediation of the adhesion to surfaces of other organisms or of non-living matter and many more.

All the biopolymers are synthesized by enzymatic processes in the cytoplasm, in the various compartments or organelles of cells, at the cytoplasmic membrane or at cell wall components, at the surface of cells or even extracellularly, synthesis of a biopolymer may be initiated in one part of a cell and may be continued in another part as it occurs, for example,

during the synthesis of complex cell wall constituents in bacteria. (Madigan M.T., et al., 2001; Bartnicki G.S., 1968.)

Biopolymers can be obtained from agriculture or from biotechnological processes and are therefore, in principle, available from renewable resources. Autotrophic microorganisms may also be candidates for the synthesis of some of these biopolymers; however, as yet only weak perspectives for a biotechnological production of biopolymers have been outlined. (Asada Y., et al., 1999)

3.5. Production of biopolymers

There are different ways to produce biopolymers in order to make them available for interesting technical applications

1. Many biopolymers occur abundantly in nature and are isolated from plants and algae which grow in natural environments. Agar and alginates are isolated from red algae belonging to the genus Gelidium or from various brown algae also referred to as seaweeds.
2. Few biopolymers are isolated from extremely natural sources. An example of such an exception is hyaluronic acid which is extracted from the umbilical cords of new born children.
3. *In vitro* synthesis of biopolymers with isolated enzymes in cell-free systems offers another possibility to produce biopolymers. One example is the application of the heat stable DNA polymerases in the polymerase chain reaction (PCR) to produce monodisperse defined DNA molecules. Another example is dextran, which can be produced on a technical scale with isolated dextran sucrase.
4. Fermentative production of biopolymers is used in industry –example is polysaccharides.

3.5.1 Intracellular versus extracellular production of biopolymers

The biotechnological production of biopolymers may occur intracellularly or extracellularly. This causes several severe consequences regarding the limitations of the production and downstream process to obtain the biopolymers in a purified state.

PHAs cyanophycin, glycogen, starch, and polyphosphate are example of biopolymers which are accumulated in the cytoplasm of cells. The availability of space in the cytoplasm

therefore limits the amount of polymer that can be produced by a cell. This is particularly relevant for fermentative production processes mostly employing microorganisms. Therefore the yield per volume is limited /determined by the cell density and the fraction of the biopolymer in the biomass.

Poly (β-D-glutamate) and many polysaccharides, such as alginates, dextran, xanthan, and microbial cellulose are examples of biopolymers which occur outside the cells, either as a result of extracellular synthesis or of excretion by the cells. For these biopolymers, the volume of the bioreactor (instead of only that of the cytoplasm) would be available to deposit the desired biopolymer. Furthermore, breakage of cells or tissues is not required and separation of the biopolymer from the other biomass is not very complex. However biotechnological processes can merely take advantage of these features since the presence of these mostly water soluble biopolymers in the medium usually cause a high viscosity in the medium, resulting in rheological problems during the fermentation process. Unfortunately, hydrophobic water-insoluble biopolymers of biotechnological interest that occur extracellularly are not known. Therefore, in practice, the amount of biopolymer produced per volume by extracellular processes is usually lower than can be obtained by intracellular processes.

Other strategies and the use of cell-free production processes, may take advantage of the features of extracellular processes. One strategy is to apply *in vitro* synthesis of biopolymers employing isolated enzymes. Another strategy is to produce the constituents of polymers as monomers by fermentative processes and to polymerize these components subsequently by solely chemical processes. Both these strategies have already entered reality and many different examples of scale have been demonstrated (i.e., not only at the laboratory scale but also at the technical scale) Polylactide acid, for example has been produced on a large scale by such a combined biotechnological and chemical approach. (Alexander Steinbuchel et al., 2001)

One of the latest and most interesting examples is chitin and its derivative chitosan. These polysaccharides are found in a wide range of natural sources, such as crustaceans, insects, annelids, mollusks, marine invertebrates, coelenterates and also a common constituent of fungal cell walls and it is non toxic, non allergenic, anti microbial and biodegradable.

3.6. CHITOSAN

Chitosan was first discovered in 1811 by Henri Braconnot, director of the botanical garden in Nancy, France. Braconnot observed that a certain substance (chitin) found in mushrooms did not dissolve in sulphuric acid. Over the last 200 years, the exploration of chitosan has taken on many different forms. Several other researchers continue to build on the original finding of Braconnot, discovering new uses for chitin as they find different forms of it in nature.

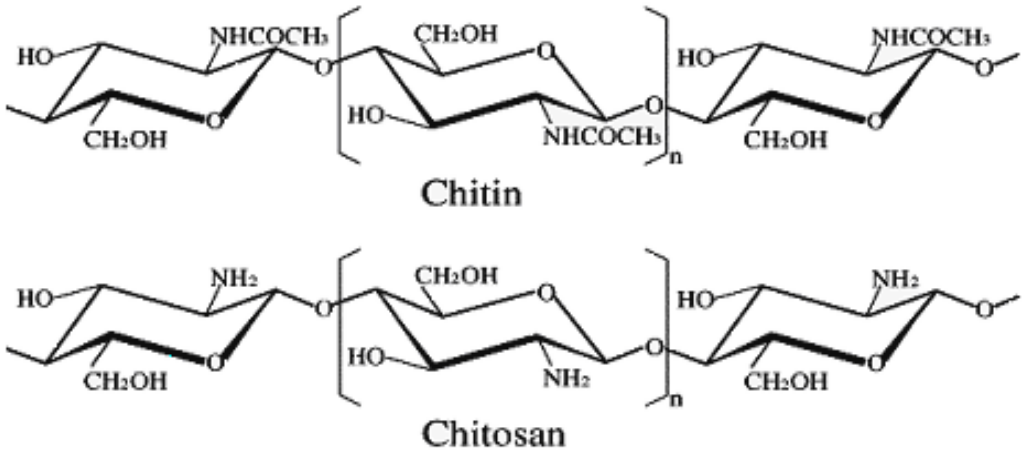


Fig. 1: Structure of Chitin and Chitosan

As seen in above Figure 1, both chitin and chitosan have similar chemical structure. Chitin is made up of a linear chain of acetylglucosamine groups while chitosan is obtained by removing enough acetyl groups (CH_3-CO) for the molecule to be soluble in most diluted acids and the only difference between chitosan and cellulose is the amine (- NH_2) group in the C-2 position of chitosan instead of the hydroxyl (-OH) group. However, unlike plant fiber, chitosan possesses positive ionic charges, which give it the ability to chemically bind with negatively charged fats, lipids, cholesterol, metal ions, proteins and macromolecules.

Chitosan is a cationic biopolymer consisting of (1,4)-linked 2-amino- deoxy-β-d-glucan is derived from chitin, a homopolymer of ß-(1-4)-linked *N*-acetyl-D-glucosamine.

Fig. 2: Marine Invertebrates

Chitin is widely available from a variety of sources among which, the principal sources are shellfish and crustacean waste materials. Industrially, Chitosan is derived from the chemical deacetylation of chitin prepared from crab or shrimp shell. However, this process fails to produce chitosan of uniform quality. The process of deacetylation involves the removal of acetyl groups from the molecular chain of chitin, leaving behind a compound (chitosan) with a high degree chemical reactive amino group ($-NH_2$). This makes the degree of deacetylation (DDA) is an important property in chitosan production as it affects the physicochemical properties and this value also differ based on the crustacean species and preparation methods used. Hence the degree of deacetylation determines its appropriate applications. Deacetylation also affects the biodegradability and immunological activity of chitosan. (Arcidiacono S., et al., 1992: Jin woo lee et al., 2003; Davidson G.R., et al., 2003)

Chitosan has attained increasing commercial interest as suitable resource material due to its excellent properties like biocompatibility, biodegradability, adsorption, ability to form films and to chelate metal ions.

3.6.1. Physico chemical properties

3.6.1.1. Physical properties

It is off white amorphous translucent flake or powder with pearly color.

3.6.1.2. Solubility

Chitin is insoluble in most organic solvents, chitosan is readily soluble in dilute acidic solutions below pH 6.0. Organic acids such as acetic, formic and lactic acids are used for dissolving chitosan. The most commonly used is 1% acetic acid solution at about pH 4.0 as a reference. (Zamani A., et al ., 2007)

Water soluble chitosan: Ordinary chitosan is insoluble in water and only soluble in some diluted organic acid and inorganic acid, which limits its application. Water soluble chitosan is refined with special techniques, improving the soluble performance of ordinary chitosan and retaining the macromolecule performance thereby widening the scope of its application and increasing its convenience of use.

3.6.1.3. Degree of Deacetylation (DDA) (Tanverr A., et al., 2002)

The degree of deacetylation can be employed to differentiate between chitin and chitosan because it determines the content of free amino groups in the polysaccharides. The degree of deacetylation is an important parameter associated with the physical and chemical properties of chitosan, because it is directly linked to the chitosan cationic properties.

The degree of deacetylation of chitosan ranges from 56% to 99% with an average of 80%, depending on the crustacean species and the preparation methods. Various methods have been reported for the determination of the degree of deacetylation of chitosan. These included Ninhydrin test, linear potentiometric titration, near-infrared spectroscopy, nuclear magnetic resonance spectroscopy, hydrogen bromide titrimetry, infrared spectroscopy, and first derivative UV-spectrophotometry. (Chan H.Y., et al., 2005)

3.6.1.3.1. Determination of degree of deacetylation by Infrared spectroscopy (Muzzareli et al., 1997, Brugnerato J., et al., 2001)

The IR spectroscopy method, which was first proposed by Moore and Roberts (1980), is commonly used for the estimation of chitosan Degree of Deacetylation. This method has a number of advantages and disadvantages,

- First, it is relatively fast and unlike other spectroscopic methods, does not require purity of the sample to be tested nor require dissolution of the chitosan sample in an aqueous solvent.
- However, the IR method utilizing baseline for DDA calculation, and as such there may be

possible argument for employment of different baseline which would inevitably contribute to variation in the DDA values.

- The sample preparation, type of instrument used and conditions may influence the sample analysis. Since chitosan is hygroscopic in nature and samples with lower DDA may absorb more moisture than those with higher DDA, it is essential that the samples under analysis be completely dry.

The following are some baselines proposed for the determination of the degree of deacetylation of chitosan

i. Domszy and Roberts (1985), DDA = 100-[(A1655 / A3450) x 100/1.33]
ii. Sabnis and Block (1997), DDA = 97.67-[26.486X (A1655 / A3450)]
iii. Baxter et al (1992), DDA = 100 – [(A1655 / A3450) X 115]
iv. Rout (2001), DDA = 118.883-[40.1647 X (A1655/A3450)]

3.6.1.4 **. Molecular Weight**

Chitosan is a biopolymer of high molecular weight. Like its composition, the molecular weight of chitosan varies with the raw material source and method of preparation. Molecular weight of native chitin is usually larger than one million Daltons while commercial chitosan products have the molecular weight range of 100,000 – 1,200,000 Daltons, depending on the process and grades of the product.

3.6.1.5. Purity

The purity of the product is vital particularly for high-value product (biomedical or cosmetic area) purity is quantified as the remaining ashes, proteins, insolubles and also in the Bio-burden (microbes, yeasts and moulds, endotoxins). Even in the lower value chitosan such as that used for the waste water treatment, the purity is a factor because the remaining ashes or proteins tend to block active sites, the amine grouping. Application of chitosan can be classified mainly in three categories according to the requirement on the purity of chitosan:

- Technical grade for agriculture and water treatment
- Pure grade for the food and cosmetics industries
- Ultra-pure grade for biopharmaceutical uses

3.7. Chitosan production by traditional method

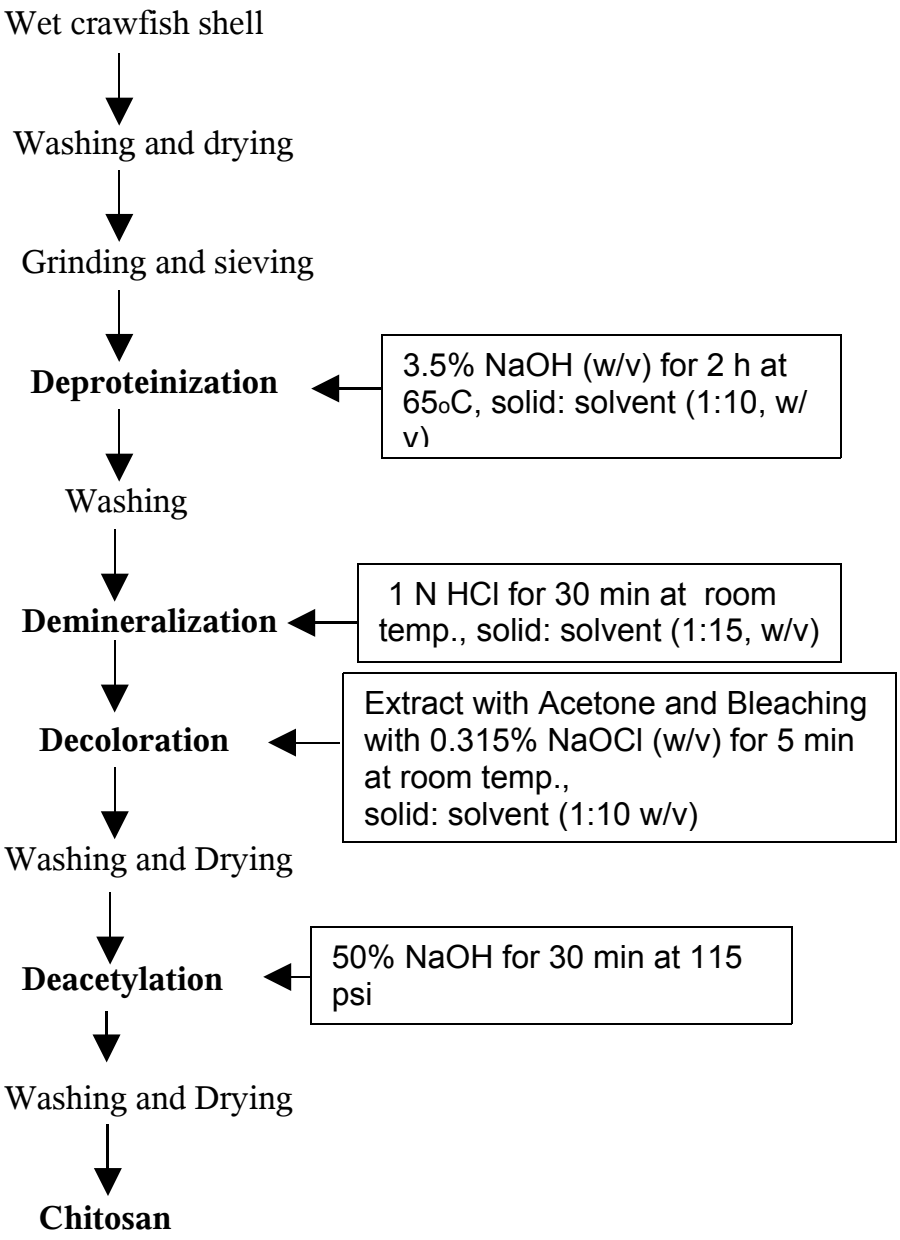


Fig. 3: Flow scheme of traditional chitosan production

(Jin woo lee et al., 2003)

3.8. FUNGAL PRODUCTION OF CHITOSAN

An alternative to solve the above described problems related to traditional chitosan production is the fungal production of chitosan, which contain chitin and chitosan as cell wall component. The amount of these polysaccharides depends on the fungi species and culture

conditions. The main advantage of fungal production of chitosan is that it is eco friendly, independent of seasonal factor, possibility of wide scale production, simultaneous extraction of chitin and chitosan, easy handling, harvesting, absence of protein contamination and controlling the production of high quality of chitosan. The physicochemical properties and yield of chitosan isolated directly from a fungus may be optimized by controlling the fermentation and processing parameters. Fungal biomass can be produced by solid state/substrate fermentation (SSF) and submerged fermentation (SmF). The fungal mycelial wastes of the either antibiotic or citric acid industry can also serve as a rich alternative source for chitosan production. (New N., et al., 2007; White SA., et al., 1979)

3.8.1. Architecture of fungal cell wall:

The fungal cell wall is a complex dynamic assemblage of many components. In sections of fungal cells viewed by electron microscopy the wall typically appears to be composed of layers although it has recently become clear that the layers are not distinctly, but interconnected as one massive multimolecular complex.

3.8.2. Composition

Approximately 80% of the cell wall of fungi consists of polysaccharides. Most fungi have a fibrillar structure built on chitin, chitosan and ß-glucans, and a variety of heteropolysaccharides. The fibers are contained in a complex gel-like matrix. Proteins constitute a small fraction of wall material, rarely more than 20%, and often as glycoprotein. Not all proteins have a structural role. Mating, recognition, wall modification and nutrition involve wall-bound proteins.

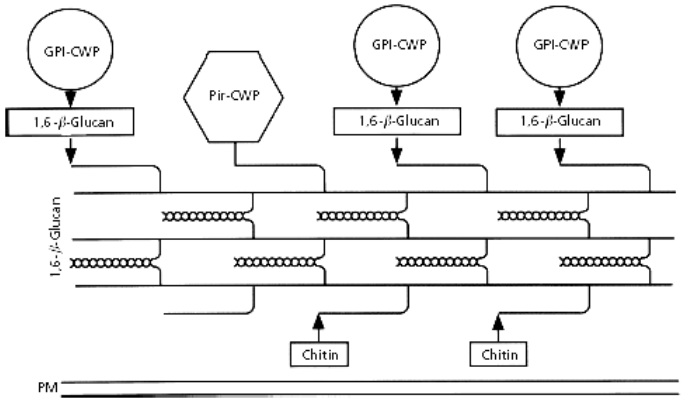


Fig. 4: Molecular architecture of the yeast cell wall

The constituents of cell walls are synthesized in the cytoplasm, linked to the walls of the hyphal tip, and polymerized and cross-linked in the wall matrix. Chitin (structure of the monomer shown in above figure 4) and the glucans are synthesized at the plasma membrane by enzymes embedded in the membrane. Nucleotide sugar precursors are accepted from the cytoplasm, linked and passed to the wall. Wall glycoproteins are synthesised in the endoplasmic reticulum, carried through the golgi to the plasma membrane, where vesicles release the glycoprotein to the wall. Enzymes cross-linking fibrils in the wall are released through the plasma membrane.

3.9. Biosynthesis of chitosan

Studies on a variety of fungi have demonstrated the enzymes and biosynthetic steps involved in the conversion of glucose -6-phosphate into the chitin precursor uridine diphosphate N-acetylglucosamine, UDP-GlcNAc. A single enzyme activity is involved in the final step, chitin synthase. Chitin deacetylase enzyme is involved in the conversion of chitin to chitosan.

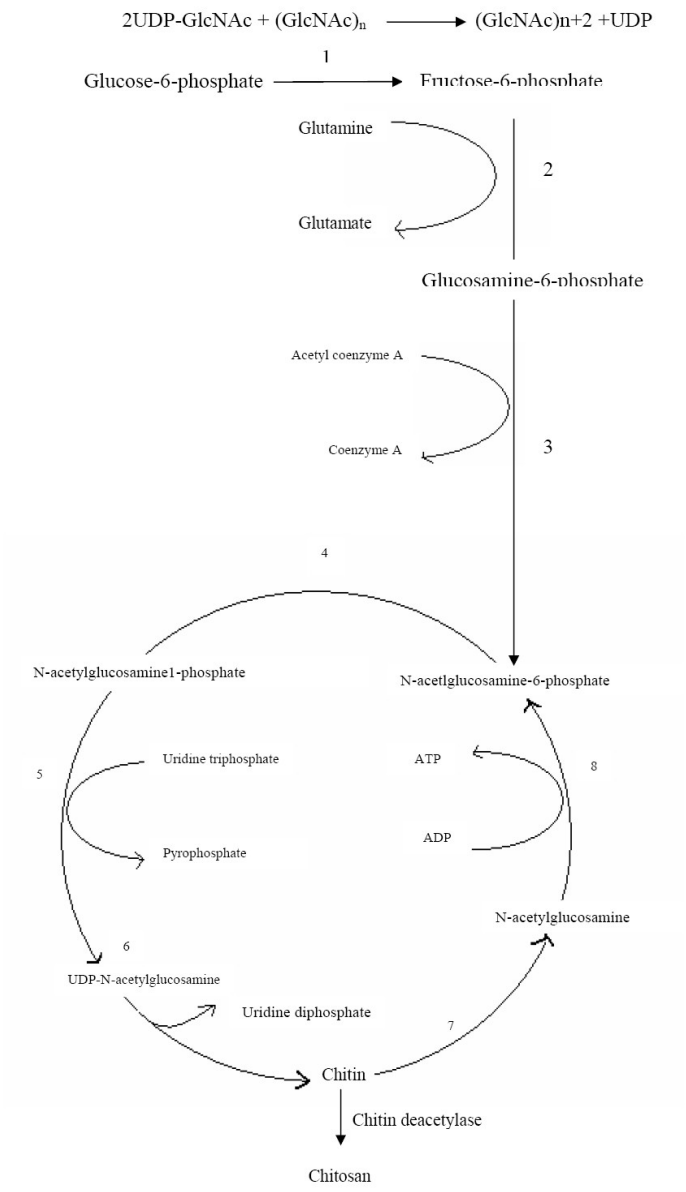


Fig. 5 Pathway of chitosan synthesis (▶) and N- acetylglucosamine cycle ( )

Chitin deacetylase

:etylase

follows :1,Phosphoglucoisomerase:2,Glucosamine 6-phosphate synthase; 3, Glucosamine-phosphate-acetyltransferase; 4, N- acetylglucosamine phosphomutase; 5, UDP-N- acetylglucosamine pyrophosphorylase; 6, Chitin synthase; 7, Chitinase and N-acetylglucosaminidase: 8, N-acetylglucosamine kinase. (Carlile M.J., et al., 2001)

3.10. Antimicrobial Properties of chitosan

Recent studies on the antibacterial activity of chitosan have revealed that chitosan is effective in inhibiting growth of bacteria and it depends on its molecular weight and the type of bacterium. (Chen C., et al., 1998)

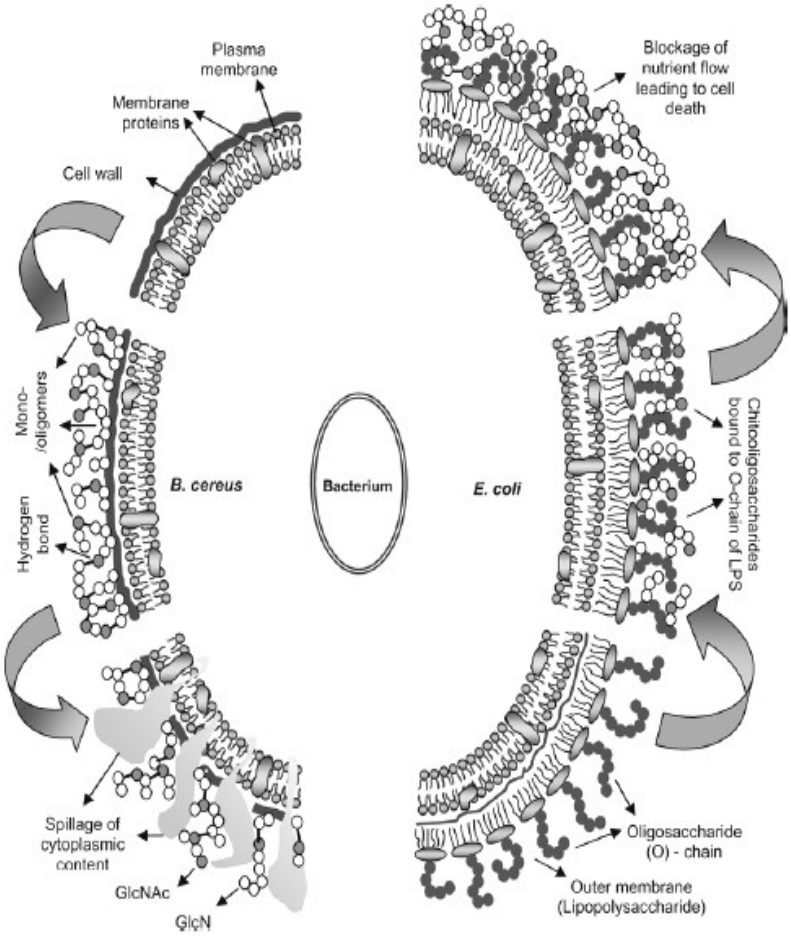


Fig.6: Mechanism of antimicrobial activity of chitosan

Mechanism of bactericidal action The mode of action of cationic antibacterial agents is widely believed to be due to interacting with and disrupting the wall membrane structure.

3.13. Antioxidants

Antioxidants are molecules which can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. Although there are several enzyme systems within the body that scavenge free radicals, the principle micronutrient (vitamin) antioxidants are vitamin E, beta-carotene, and vitamin C. Additionally, selenium, a trace metal that is required for proper function of one of the body's antioxidant enzyme systems, is sometimes included in this category. The body cannot manufacture these micronutrients so they must be supplied in the diet.

Types of Antioxidants

Antioxidants can be classified into the following types:

1. Primary antioxidants
2. Secondary antioxidants
3. Tertiary antioxidants

Primary antioxidants

They prevent the formation of new radical species either by converting existing free radical units to harmless molecules or by preventing formation of free radicals from other molecules. E.g., Superoxide dimutase, glutathione peroxidase and metal binding proteins.

Secondary antioxidants

They trap free radicals thereby preventing chain reactions. E.g., Vitamin E, Vitamin C, β-carotene, uric acid and albumin.

Tertiary antioxidants

They repair biomolecules damaged by free radicals. E.g., Methionine sulphoxide reductase.

Sources of Antioxidants

- Allium sulphur compounds: onion and garlic
- Anthocyanins: grapes and berries
- β carotene: pumpkin, mangoes, apricots, carrots and spinach
- Flavonoids: green tea, citrus fruits, red wine, onion and apples
- Isoflavonoids: soyabeans, lentils, peas and milk; Lutein: spinach
- Polyphenols: thyme;Selenium – seafood, lean meat and whole grains
- Vitamin C: oranges, mangoes, spinach and strawberries

Therapeutic Applications of Antioxidants

Antioxidants scavenge the free radicals from the body cells and prevent or reduce the damage caused by oxidation. Diets high in antioxidants may also be associated with a decrease risk of breast cancer.

Studies have shown that antioxidant therapy improves responses to therapeutic drugs and stop fibrinogenesis in people with hepatitis of various cases. Antioxidants have been shown to raise CD 4^+ cells. This is considered an important goal for the management of HIV.

In heart disease and stroke, it is possible that higher levels of antioxidants slow or prevent the development of arterial blockages, a complicated process involving the oxidation of cholesterol.

Antioxidants are effective scavenges of super oxide and other oxygen reactive species and they prevent endothelial dysfunctions caused by hyperocysteinemia. Vitamin E potentially provides additional risk reduction in retinopathy and nephropathy in addition to those achievable through insulin therapy. Therefore, antioxidants can be considered as potential prophylactic and therapeutic agents for a number of disease conditions.

IV. REVIEW OF LITERATURE

- Kim et al., (2007) had reviewed chemical modification of chitosan as a gene carrier *in vitro* and *in vivo.* Currently, the success of gene therapy is mainly limited due to the lack of effective vector systems. Chitosan has been investigated as a non-viral vector. The low transfection efficiency and low cell specificity of chitosan as a DNA carrier needs to be overcome before undertaking clinical trials. This review summarizes the use of chitosan and chitosan derivatives in gene therapy, and particularly the role of several factors for the enhancement of transfection efficiency and cell specificity *in vitro.*

- Yena et al., (2007) had reported the antioxidant properties of fungal chitosan from shiitake stipes. Fungal chitosan B or C was prepared by alkaline N-deacetylation of crude chitin B or C for 60, 90 and 120 min, which was obtained from air-dried shiitake stipes and its antioxidant properties studied. Chitosan showed antioxidant activities of 61.6–82.4% at 1 mg/ml and showed reducing powers of 0.42–0.57 at 10 mg/ml. At 10 mg/ml, scavenging abilities of chitosan B60 and C60 on 1,1-diphenyl-2- picrylhydrazyl (DPPH) radicals were 28.4–31.3% whereas those of chitosan B90, B120, C90 and C120 were 44.5–53.5%. With regard to the scavenging ability on hydroxyl radicals at 0.1 mg/ml, chitosan B60 and C60 were 61.9% and 63.6%, chitosan B90 and C90 were 68.3% and 69.9% and chitosan B120 and C120 were 77.7% and 77.2%, respectively. At 1.0 mg/ml, chelating abilities of chitosan B60 and C60 on ferrous ions were 88.7–90.3% whereas those for the other chitosan were 97.8–103%. EC_{50} values of antioxidant activity were below 1 mg/ml whereas reducing power and scavenging abilities on DPPH radicals were 7.69–16.3 mg/ml. EC_{50} values of scavenging abilities on hydroxyl radicals were below 0.1 mg/ml whereas chelating abilities of ferrous ions were 0.58–0.69 mg/ml.

- Xiaohui Wang et al., (2007) had reported preparation, characterization and antimicrobial activity of chitosan–Zn complex. Five chitosan–zinc complexes with different zinc content were prepared and characterized by FT-IR, XRD, AAS and elemental analysis. *In vitro* antimicrobial activities of complexes were evaluated against 11 species of bacteria and fungi. The complexes showed effective and wide spectrum antimicrobial activity. The complexes exhibited better antibacterial activity than antifungal activity.

- Zamani et al., (2007) had reported extraction and precipitation of chitosan from cell wall of zygomycetes fungi using dilute sulphuric acid. Chitin is insoluble in neither cold nor hot dilute sulphuric acid. Similarly chitosan is not soluble at room temperature but is dissolved in 1% H_2SO_4 at 121^0 C within 20 min. The new method was developed to measure the chitosan content of the biomass and cell wall of fungus *Rhizomucor pusillus* and results indicated 8% of the biomass as chitosan.

- Di Mario et al., (2007) had reported isolation of chitin and chitosan isolated from the mycelium of seven species of Basidiomycetes and evaluated the possibility of using fungal biomass as a source of chitin and chitosan. The material was characterized for its purity and degree of acetylation. Chitin yields ranged between 8.5 and 19.6% dry weight and the chitosan yield was approximately 1%. The characteristics of the fungal chitins were similar to those of commercial chitin.

- Nwe et al., (2007) had reported the decomposition of mycelial matrix and extraction of chitosan from *Gongronella butleri* USDB 0201 and *Absidia coerulea* ATCC 14076. Free chitosan, 2g/100g mycelia from *Gongronella butleri* and 6.5g/100g mycelia from *Absidia coerulea* were isolated by 1M NaOH at 45 degrees C for 13h and 0.35M acetic acid at 95 degrees C for 5h. According to these results, *G. butleri* has higher amount of complexed chitosan and *A. coerulea* has higher amount in free chitosan.

- Chatterjee et al., (2007) had described that enhancement of growth and chitosan production by *Rhizopus oryzae* in whey medium by plant growth hormones, at different concentrations increase the mycelial growth by 19-32%. However, increase in chitosan content of the mycelia was relatively small (1.7-14.3%) compared to the control. Maximum enhancement was observed with gibberellic acid. Hormones, at higher dose, instead of stimulation inhibited both growth and mycelial chitosan content.

- Rabea et al., (2006) had studied, enhancement of fungicidal and insecticidal activity by reductive alkylation of chitosan. In this study series of N-alkyl chitosan (NAC) derivatives were synthesized and examined for fungicidal and insecticidal activity. The chemical structures were characterized by IR and ^1H NMR spectroscopy. Their fungicidal activity

was evaluated against the grey mould *Botrytis cinerea* Pers and the rice leaf blast pathogen *Pyricularia grisea* Sacc by a radial growth bioassay. It was of interest that most of the NAC derivatives were more active against both fungi than chitosan itself.

- ☞ Nakashima et al., (2006) had studied the mechanical properties and antibacterial efficacy of chitosan films. Chitin, chitosan and quaternary chitosan films were prepared, and evaluated. The antibacterial activities of quaternary chitosan films against Staphylococcus aureus and *Escherichia coli* were stronger than those of chitosan films.

- ☞ Chatterjee et al., (2005) had reported the production and physico-chemical characterization of chitosan from *Mucor rouxii*: Fungal culture media and fermentation condition can be manipulated to provide chitosan of more consistent physico-chemical properties compared to that derived chemically from chitin using three different media, viz., molasses salt medium, Potato Dextrose Broth and yeast extract peptone glucose medium under submerged condition. Their physico-chemical properties such as ash, moisture, protein contents and specific rotation did not show much difference.

- ☞ Liu et al., (2004) had reported that chitosan kills bacteria through cell membrane damage. The bactericidal activity of chitosan acetate solution (CS) against *Escherichia coli* and *Staphylococcus aureus* was evaluated. Morphologies of bacteria treated with CS were observed by transmission electron microscopy (TEM). Results showed that CS increased the permeability of the outer membrane (OM) and inner membrane (IM) and ultimately disrupted bacterial cell membranes, with the release of cellular contents. This damage was likely to be caused by the electrostatic interaction between NH_3^+ groups of CS acetate and phosphoryl groups of phospholipid components of cell membranes.

- ☞ Niederhofer and Muller. (2004) had described the method for direct preparation of chitosan of low molecular weight from fungi. By modifying the common method for the preparation of chitosan from fungi, low molecular weight chitosan with an average MW of 4.5×10^4 g/ mol and a numerical MW of 1.7×10^4 g/mol could be directly extracted from the raw material without the need of thermal or chemical depolymerization. Based on the solubility of low molecular weight chitosan up to alkaline pH ranges, reprecipitation and washing

with ethanol is required to keep the low molecular fraction within the preparation. The use of water for washing between the preparation steps would cause solving and discarding of the low molecular chitosan. The chitosan was analyzed by laser light scattering and ^{1}HNMR spectroscopy.

- New and Stevens. (2004) had reported the effect of urea on fungal chitosan production in solid substrate fermentation. The fungus *Gongronella butleri* USDB 0201 was grown on sweet potato pieces supplied with different amounts of urea at 26 ^{0}C for 7 days. Crude chitosan was extracted and treated with amylolytic enzyme to remove bound glucan. The distribution of the molecular weight of the chitosan was studied by gel exclusion chromatography. The conditions for optimal production of fungal chitosan by solid substrate fermentation were investigated. The initial pH and the amount of urea influence the yield of fungal mycelia and chitosan.

- Yoshihara et al., (2003) reported chitosan productivity enhancement in *Rhizopus oryzae* YPF-61A by D-psicose. The effect of the rare sugar D-psicose on chitosan production by *Rhizopus oryzae* was studied. The fungus was not able to utilize D-psicose as a sole source of carbon, either for germination of the spores or for growth of the vegetative cells. In a medium containing low amount of D-glucose, however, D-psicose supplementation between 5 and 12 g/l enhanced of the productivity of chitinous substances especially chitosan, in the cell walls.

- Lavertu et al., (2003) had reported a validated ^{1}HNMR method for the determination of the degree of deacetylation (DDA) of chitosan. The precision, ruggedness, robustness, specificity, stability and accuracy of the technique are discussed in this paper.

- Pochanavanich and Suntornsuk. (2002) studied the production and characterization of fungal chitosan. Chitosan production by several species of fungi, including two yeast strains namely *Zygosaccharomyces rouxii* and *Candida albicans*, were investigated for their ability to produce chitosan in complex media. Fungal chitosan had a degree of deacetylation of 84-90% and a molecular weight of 2.7 x 104-1.9 x 105 Da with a viscosity of 3.1-6.2 centipoises (cP). *Rhizopus oryzae* TISTR3189 was found to be the maximum producer of

chitosan.

- Chen et al., (2002) had studied the antibacterial properties of chitosan against waterborne pathogen. Chitosan exhibited the highest antibacterial activity against *Pseudomonas aeruginosa* on the solid agar and in liquid broth. The higher deacetylation degree and higher concentration of chitosan resulted in greater antibacterial activity.

- Tianwei et al., (2002) had reported the separation of chitosan from Penicillium chrysogenum mycelium and its applications. Alkaline treatment and acetic acid extraction were used for deacetylating chitin to chitosan. The molecular weight of the chitosan was 3.6 x 10^4 and the degree of deacetylation was 84%. Analysis indicated that the chitosan was similar to that obtained from shrimp and was used to prepare microcapsules for controlled drug release.

V. MATERIALS AND METHODS

5.1. APPARATUS

Assay Petri dishes	-	Anumbra
Centrifuge tubes	-	Borosil
Glass pipettes	-	Borosil
Micropipettes	-	Vari Pipettes
Sterile discs	-	Hi Media
Sterile swab	-	Hi Media
Standard flask	-	Borosil
Test tubes	-	Borosil

Ingredients	**Manufacturers**
Acetic acid	Qualigens, Mumbai
Acetone	Qualigens, Mumbai
Agar-Agar	Himedia labs ltd,Mumbai
Ammonium sulphate	S.d fine chemicals ltd,Mumbai
Arginine	Loba chemie pvt. Ltd
Calcium chloride	S.d fine chemicals ltd,Mumbai
Corn starch	Himedia labs ltd,Mumbai
Corn steep liquor	Sigma-Aldrich
Dextrose	Loba chemicals
DPPH	Sigma-Aldrich, Germany
Ethanol	Qualigens, Mumbai
Glucose	S.d fine chemicals ltd,mumbai
Magnesium sulphate 7 H_2O	S.d fine chemicals ltd,Mumbai
Maltose	Nice chemicals, Cochin
Maltose	Loba chemie pvt. Ltd
Methanol	Qualigens, Mumbai
Non absorbent cotton	Ramaraju surgical cotton ltd
Peptone	Himedia Labs ltd,Mumbai
Ponceau S	Loba Chemie pvt. Ltd
Potassium bromide	S.d Fine Chemicals Ltd,Mumbai
Potassium chloride	S.d Fine Chemicals Ltd,Mumbai
Potassium phosphate dibasic	Loba Chemie Pvt. Ltd
Sodium chloride	S.d Fine Chemicals Ltd,Mumbai
Sodium hydroxide	Hi-Pure Fine Chem. Ltd Chennai
Soy bean meal	Himedia Labs Ltd,Mumbai
Stains -all	Sigma-Aldrich, Ugraine
Sucrose	Commercial
Tween 20	Loba Chemie Pvt. Ltd

Tween 80	Loba Chemie Pvt. Ltd
Yeast extract powder	Himedia Labs Ltd,Mumbai

5. INSTRUMENTS

Instruments	**Company**
Hot air oven	Technico
Incubator	Technico
Autoclave	Universal autoclave
Conical flask	Borosil
Centrifuge	Eppendorf
Compound microscope	Motic
Digital balance	Shimadzo
Heating mantle	Guna enterprises
vertical laminar flow hood	Technico
Digital Orbital shaker	Remi motors
Micro pipettes	Vari pipettes (Hi –Tech lab)
Micro tips	Tarsons

5.3. CULTIVATION MEDIA AND CONDITIONS

Maintenance media

All the strains obtained from National collection of Industrial Microorganisms, National Chemical Laboratory, Pune, India. The cultures were maintained on a Potato Dextrose Agar slant. This culture was transferred once a month to a fresh slant.

5.4. Identification of fungi by microscopy: (Aneja K.R., et al., 2001)

Scotch tape preparation for studying morphology of fungi

This is a rapid technique for preparing a temporary microscopic mount of a fungus without disturbing the arrangement of conidia and conidia bearing hyphae, the conidiophores.

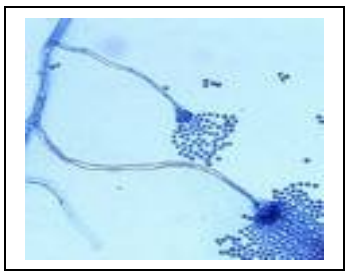
Requirements

Fungus colony on agar plate, Strip of clear cello tape 10 cm, Lactophenol cotton blue, Microscopic slide.

Procedure:

1. A clean slide was taken and a drop of lactophenol cotton blue was placed in the centre of the slide.
2. The Tape was held the tape with sticky side down, between thumb and forefinger of each hand and pressed firmly.
3. The centre of the sticky side of the tape was pressed firmly on to the surface of the fungus colony, where sporulation was visible.
4. The tape was gently pulled away from the colony and placed on the drop of lactophenol cotton blue.
5. The extended ends of the tape were folded over the ends of the slide.

Observations:



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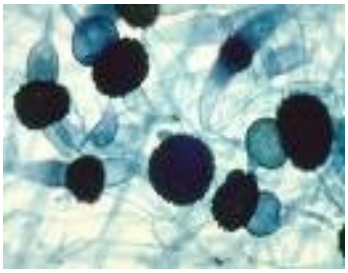
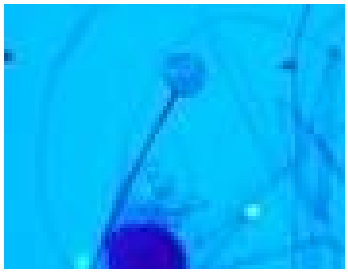




3

Fig.12: Lacto phenol cotton blue staining (40x)

1. *Aspergillus niger*
2. *Mucor hiemalis*
3. *Rhizopus oryzae*

5.5. Identification of chitosan containing fungi (My lien Dao. 2007)

This method is a novel method of staining for the detection of chitosan containing fungi and protozoa under a light microscope.

Requirements

This staining method comprises five reagents

1. 0.1% Ponceau S in 5% acetic acid in water.
2. 0.2% stains all in methanol (stock solution), which is diluted 1:10 in a solution of acid Methanol (solution 4) before use.
3. 0.25% sodium dodecyl sulphate in a phosphate buffered saline.
4. Acid methanol (50:10:40 deionized water: Acetic acid: Methanol)
5. Solution of PBS containing 0.05% Tween 20 and microscope.

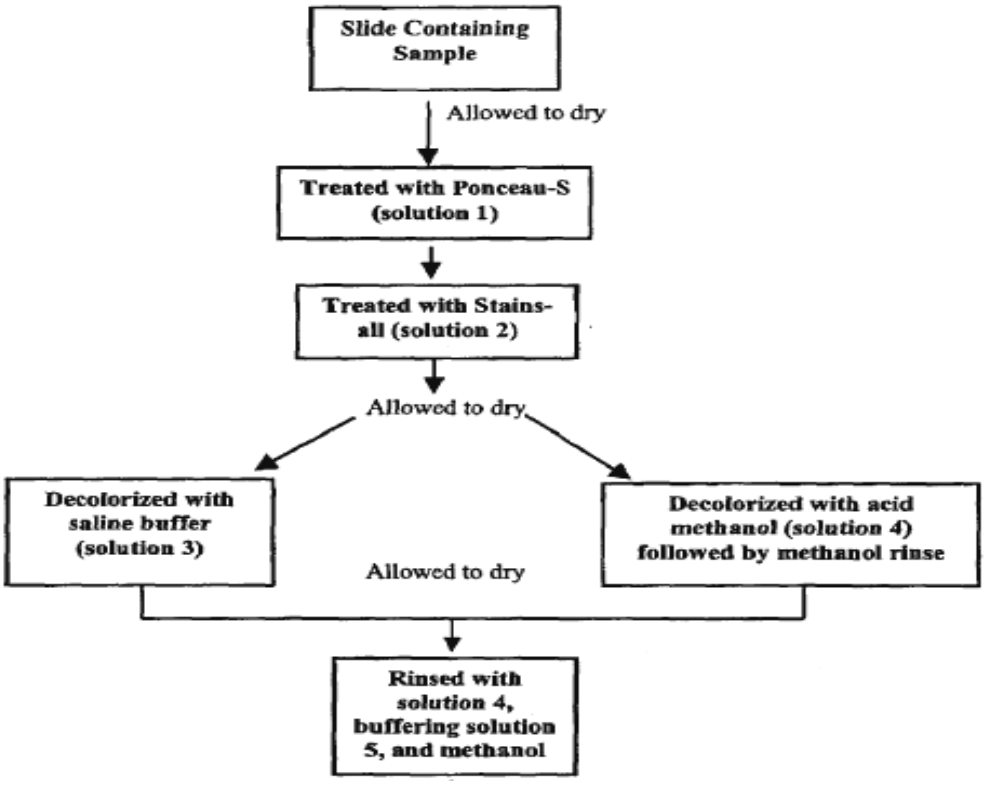


Fig. 13: Flow diagram demonstrating method of staining

Observation

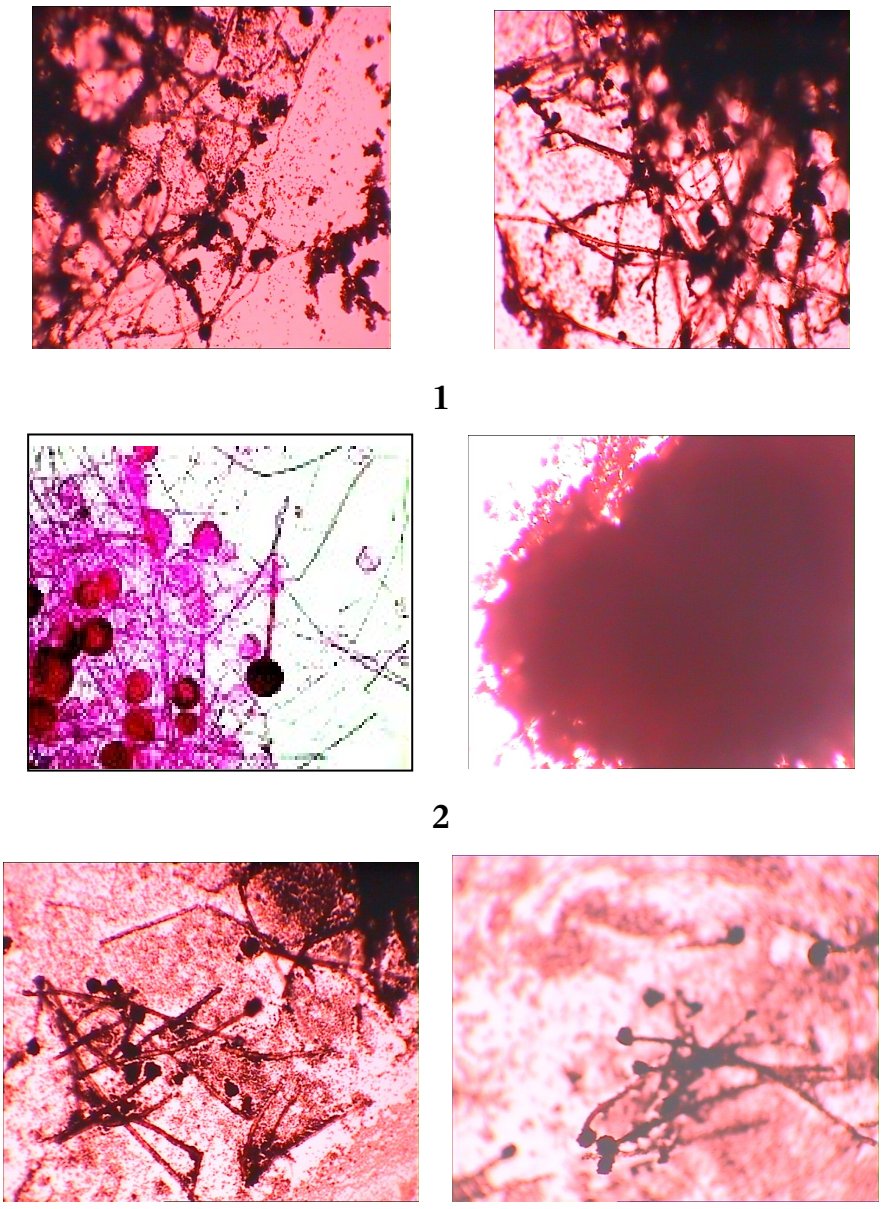


Fig. 14: Photographic slides of the chitosan staining in fungi (40x)

1. *Aspergillus niger*

2. *Mucor hiemalis*

3. *Rhizopus oryzae*

5.6. Inoculum preparation

Whenever required, all cultures were subcultured on Potato Dextrose Agar plates, incubated at room temperature (25°C) and exposed to black light to stimulate sporulation. The cultures were allowed to grow for 3-5 days for spores formation. Spores were harvested by flooding the culture plates with 5% Tween 80 in sterile distilled water. A final spore suspension ($1.8 \times l0^6$ spores/ml) was prepared for each fungal species and used to inoculate the fermentation broth.

5.7. Fermentation medium (Chen M.H., et al., 2001)

The fermentation medium contains 20g/L of glucose, 10 g/L of peptone, 1g/L of yeast extract, 5 g/l of ammonium sulphate, 1 g/L of a K_2HPO_4, 1 g/L of $MgSo_4 .7H_2O$, 0.1 g/L calcium chloride and sodium chloride 1g/L. After inoculation the fungi was grown in the fermentation broth for an additional two days in a shaking incubator set at 28˚ C with agitation of 200 rpm, the pH of the fermentation medium was maintained between 3-4, throughout the fermentation . At the end of the desired incubation period the mycelia was harvested by filtration.

5.8. Chitosan isolation

The biomass was recovered from the fermentation medium by filtration (no. 1 Whatman) and washed with distilled water to get clear filtrate. The mycelium was then treated with 1 M sodium hydroxide (1:30g/ v) and the mixture was autoclaved at 121˚ C for 15 minutes. The mixture was subsequently centrifuged at 12000g for 5 minutes to sediment the alkali insoluble materials (AIM) and washed with distilled water and ethanol. The washed material was further extracted with 10% acetic acid solution (1:40g/ ml) reflexed at 60˚C for 6 hours. The resulting slurry was then isolated by centrifugation at 12,000 g for 15 minutes yielding an acid soluble supernatant (**containing chitosan**) and an acid insoluble precipitate

(**containing chitin**). The pH of the supernatant was adjusted to 10 with 4 M sodium hydroxide, thereby precipitating out the chitosan. The chitosan was finally washed with distilled water, 95% ethanol (1: 20 w/v), acetone (1:20 w/v) subsequently, and air dried. (Chen MH et al., 2001, Pochanavanich P. et al., 2002, Thayaza et al., 2007)

5.9. Determination of growth curve, extractable chitin and chitosan (Shimahara K., et al., 1998)

The growth curves, extractable chitosan and chitin of *R. oryzae, M.hiemalis* and *A.niger* were determined by culturing each fungus in the fermentation medium. This was done by inoculating 30 ml of spore inoculum in 270 ml of fermentation medium. The mycelial dry weight was determined by filtration and drying the biomass at 65^0C extractable chitosan and chitin after 24, 48, 72, 96, 120, 144, 168 and 192 hours of growth were determined as described above. Three replicate cultures were prepared for each incubation period.

5.10. Medium optimization (Chen MH., et al., 2001; Chen MH., et al., 2002)

In the media optimization for the three strains two variations in the experimental procedure was adopted.

5.10.1. Carbon source optimization

The nitrogen source i.e. peptone and mineral sources used earlier was kept as a constant and various carbon sources, namely dextrose, maltose, sucrose and corn starch were used.

5.10.2. **Nitrogen source optimization**

The carbon source i.e. glucose and mineral sources used earlier was kept as a constant and various nitrogen sources, namely soybean meal, corn steep liquor, arginine and urea were used.

5.11. Chitosan characterization

5.11.1. Viscosity (Vilai Rungsardhong et al., 2005)

The viscosity of 0.1% chitosan in 0.5% acetic acid solution was determined by using Brook Field viscometer (Version 5.1, Spindle No 62, rpm 100) at 25^0C.

Fig. 15: Brook Field DV-I viscometer

5.11.2. Infrared spectroscopy (Deacetylation degree-DDA %)

The degree of deacetylation is determined by using the absorbance ratio A1655/A3450 and calculated according to the following equation:

DDA % = 118.883-[40.1647 X (A1655/A3450)]

Two milligrams of fungal chitosan which had been dried overnight at 60˚C was thoroughly blended with 100mg of KBr, to produce 0.5 mm thick discs. Spectrum was recorded using JASCO FTIR-410 in the Pharmaceutical analysis laboratory, College of Pharmacy, SRIPMS, Coimbatore-44.

5.11.3. ^{1}HNMR (Lavertu M., et al., 2003)

^{1}H NMR measurement was carried in Bruker NMR spectrometer under static magnetic field of 300 Mhz. chitosan preparations was dissolved in D_20 and poured into 5-mm inner diameter NMR tubes. A spectrum was recorded on Bruker at CARISM department, SASTRA University, Tanjore.

5.12. Antioxidant activity by DPPH method (Lin H.Y., and Chou C.C., 2004; Yena M.T, et al., 2007 ; Mensor L.L., et al., 2001)

Principle

DPPH is a free radical, stable at room temperature, which produces a violet coloration in ethanol. It is reduced in the presence of an antioxidant molecule, giving rise to uncolored ethanol solutions. The use of DPPH provides an easy and rapid way to evaluate antioxidants.

Procedure

Each chitosan sample (0.1- 10mg/ml) in 2g/l acetic acid solution (2.5ml) was mixed with 1ml of ethanolic solution containing DPPH (Sigma) radicals, resulting in a final concentration of 10mmol/l DPPH. The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance is then measured at 518 nm against a blank.

The absorbance value was converted into the % of antioxidant activity (AA) using the following formula

$$\mathbf{AA\ (\%) = 100 - \frac{[Absorbance\ of\ sample - Absorbance\ of\ blank]}{[Absorbance\ of\ control]} \times 100}$$

Ethanol (1.0 ml) plus sample solution was used as blank. DPPH solution (1.0 ml; 10 mM) plus ethanol (2.5 ml) was used as negative control. The positive controls were those using the standard solutions.

EC_{50} value (mg/ml) is the effective concentration at which DPPH radicals is scavenged by 50% and it was obtained by interpolation from linear regression analysis. Ascorbic acid was used for comparison.

5.13. **SCREENING FOR ANTIMICROBIAL ACTIVITY** (Mackie and McCartney 1996)

Media used

Mueller Hinton agar: (For Antibacterial)

Ingredients

Peptic digest of animal tissue	- 5gms
Sodium chloride	- 5gms
Beef extract	- 1.50gmd
Yeast extract	- 1.50gms
Distilled water	- 1000ml

Preparation of Media

The ingredients were dissolved in distilled water with aid of heat; pH was adjusted to 7.2 to 7.6 by using dilute alkali /dilute acid.

Potato Dextrose agar (For antifungal)

Ingredients

Glucose	- 1%
Malt extract	- 0.3%
Yeast extract	-0.3%
Peptone	-0.5%
Agar	-2.0%
Distilled water up to	-100 ml
pH	3-5

Sterilization

20-25ml of Mueller Hinton Agar was transferred to test tubes and sealed with non-absorbent cotton. It was then autoclaved at a pressure of 15 psi (121^{o}c) for not less than15 minutes.

Organisms used

Staphylococcus aureus NCIM 2079, *Pseudomonas aeruginosa* NCIM 2036, *Escherichia coli* NCIM 2118 (Clinical isolate of *E.coli,* obtained from Sri Ramakrishna hospital, Coimbatore-44.) *Bacillus subtilis* NCIM 2063, *Micrococcus luteus* NCIM 2704, *Aspergillus niger* NCIM 545 and *Candida albicans* NCIM 3100 were procured from National Chemical Laboratory, Pune and stored in the Pharmaceutical Biotechnology Laboratory, College of Pharmacy, SRIPMS, Coimbatore-44. The strains were confirmed for their purity and identity by Gram's staining method and their characteristic biochemical reactions. The selected strains were preserved by sub culturing them periodically on nutrient agar slants and storing them under frozen conditions. For the study, fresh 24 hr broth cultures were used after standardization of the culture.

Working conditions

The entire work was done using vertical laminar flow hood so as to provide aseptic conditions. Before commencement of the work air sampling was carried out using a sterile nutrient agar plate and exposing it to the environment inside the hood. After incubation it was checked for the growth of microorganism and absence of growth confirmed aseptic working conditions.

Preparation of inoculum

The inoculum for the experiment was prepared fresh in Mueller Hinton broth for bacteria and potato dextrose broth for fungi from preserved

frozen slants. It was incubated at 37°C for 18-24 hrs and used after standardization.

Samples used : Saturated solution of isolated chitosan samples and Commercial chitosan (Sigma)

(Aspergillus niger, Rhizopus oryzae, Mucor hiemalis)

Standard used : Ciprofloxacin (5µg/disc), Fluconazole (10 µg/disc)

Vehicle used : 1 % acetic acid

ANTIMICROBIAL SCREENING BY KIRBY-BAUER METHOD

(Mackie and McCartney., 1996)

Mueller Hinton Agar and Potato Dextrose agar plates were prepared aseptically to get a thickness of 5-6 mm. The plates were allowed to solidify and inverted to prevent the condensate falling on the agar surface. The plates were dried at 37°C before inoculation. The organism was inoculated in the plates prepared earlier, by dipping a sterile swab in the previously standardized inoculum, removing the excess of inoculum by pressing and rotating the swab firmly against the sides of the culture tube above the level of the liquid and finally streaking the swab all over the surface of the medium 3 times, rotating the plates through an angle of 60° after each application. Finally the swab was pressed round the edge of the agar surface. It was allowed to dry at room temperature, with the lid closed. The sterile disc containing test drug, standard and blank were placed on the previously inoculated surface of the Mueller Hinton and Potato Dextrose agar plate and it was kept in the refrigerator for one hour to facilitate uniform diffusion of the drug. Plates were prepared in duplicate and they were then incubated for 18-24 hrs. Observations were made for zone of inhibition around the drug and compared with that of standard. All the three isolated chitosan samples were tested for antimicrobial activity in duplicates.

VI. RESULTS AND DISCUSSION

6.1. Strain identification

All the three procured strains were confirmed, its macroscopic and microscopic morphology and further work was carried out.

6.2. Chitosan identification in fungi

The chitosan containing fungi were identified under light microscope by novel staining method using Ponceau S and Stains all.

All the three procured strains were stained by Ponceau S and Stains all. It confirmed the presence of chitosan.

Isolated chitosan

(a) (b)

(c) (d)

Fig. 16: (a) Commercial chitosan ,(b) Chitosan from *A.niger* (c) Chitosan from *M.hiemalis,* (d) Chitosan from *R.oryzae.*

Graph 1: Growth curve of *Aspergillus niger, Rhizopus oryzae and M.hiemalis*

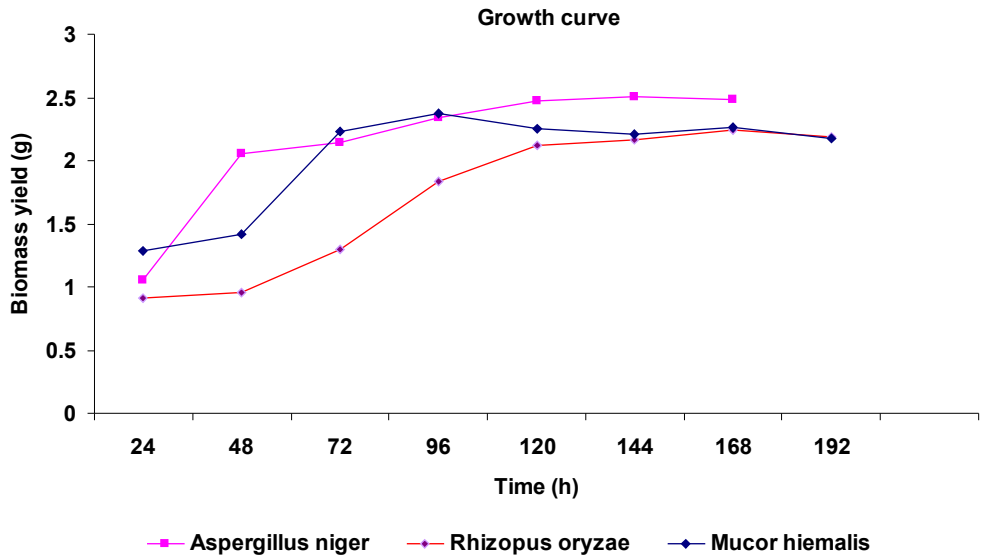


Table No 3: Weight of biomass, extractable chitosan, chitin and degree of deacetylation of *Aspergillus niger* NCIM 545

S.No	Time (h)	Weight of biomass(g)	Weight of chitosan (g)	Weight of chitin (g)	Degree of deacetylation
01	24	1.06±0.04	0.02±0.09	0.82±0.02	91.66±1.4
02	48	2.06±0.02	0.03±0.005	0.95±0.04	87.33±1.20
03	72	2.14±0.03	0.03±0.05	1.01±0.02	89±2.51
04	96	2.34±0.03	0.03±0.03	1.17±0.08	85.67±3.2
05	120	2.47±0.12	0.06±0.05	1.27±0.2	86.33±2.33
06	144	2.31±0.01	0.06±0.12	1.19±0.01	83.67±2.40
07	168	2.28±0.07	0.05±0.17	1.15±0.26	84±2.51

Values are mean ± S.E.M (n=3)

Graph no 4 : Weight of biomass, extractable chitosan, and chitin from *Aspergillus niger* NCIM 545 with incubation time.

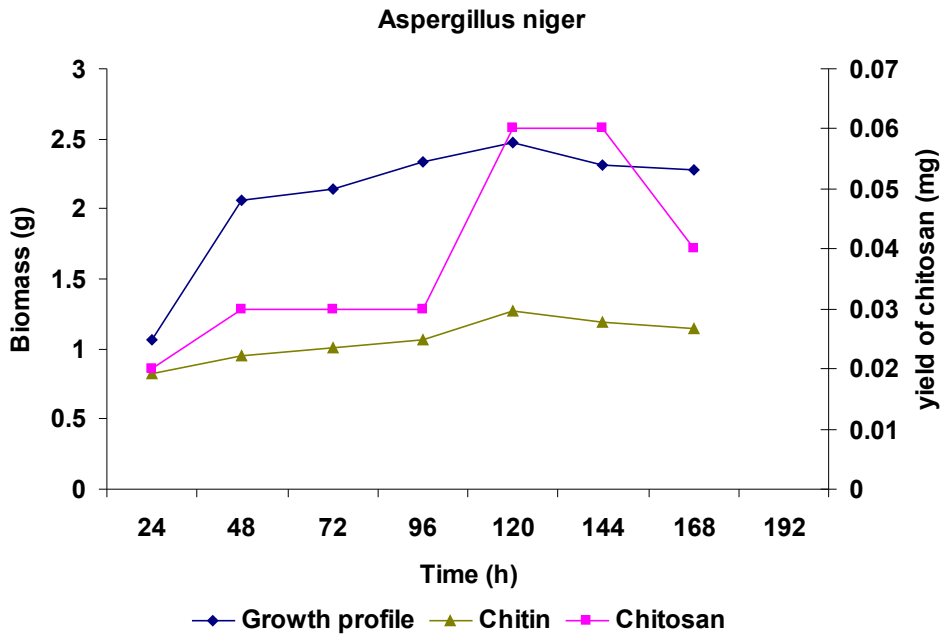


Table No 4: Weight of biomass, extractable chitosan, chitin and degree of deacetylation of *Mucor hiemalis* NCIM 873

S.No	Time (h)	Weight of biomass (g)	Weight of chitosan (g)	Weight of chitin (g)	Degree of deacetylation
01	24	1.29±0.03	0.03±0.13	0.91±0.2	91.33±2.02
02	48	1.42±0.04	0.03±0.02	0.96±0.01	84.67±1.86
03	72	2.23±0.05	0.04±0.1	1.10±0.9	90.33±1.20
04	96	2.37±0.02	0.07±0.05	1.14±0.9	90.21±2.72
05	120	2.26±0.07	0.05±0.06	1.48±0.04	84.67±1.85
06	144	2.21±0.02	0.04±0.6	1.57±0.01	89.61±2.92
07	168	2.26±0.1	0.04±0.3	1.77±0.1	88.67±0.88
08	192	2.18±0.6	0.02±0.003	1.68±0.02	84.01±2.54

Values are mean ± S.E.M (n=3)

Graph No. 3: Weight of biomass, extractable chitosan, and chitin from *Mucor hiemalis* NCIM 873 with incubation time.

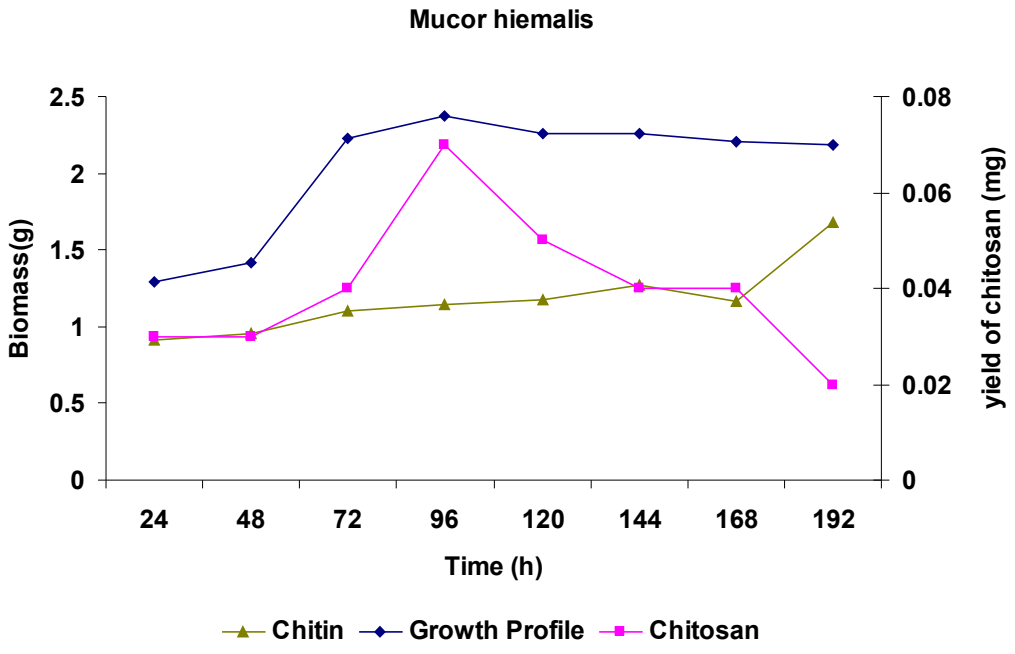
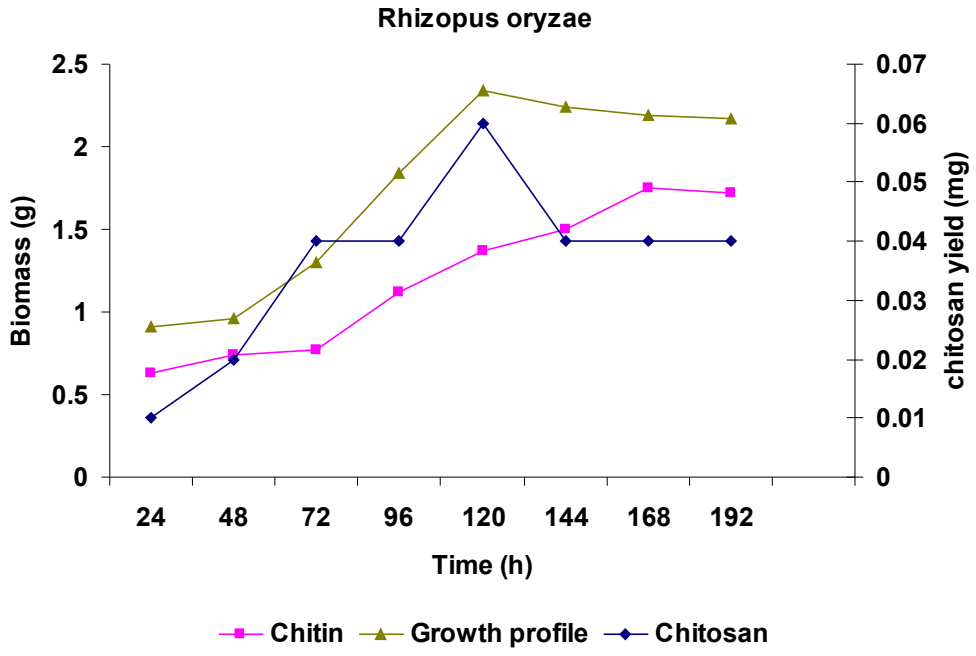


Table No 5: weight of biomass, extractable chitosan, chitin and degree of deacetylation of *Rhizopus oryzae* NCIM 879

S.No	Time (h)	Weight of biomass (g)	Weight of chitosan(g)	Weight of chitin (g)	Degree of deacetylation
01	24	0.91±0.04	0.01±0.003	0.63±0.03	90.33±1.85
02	48	0.96±0.1	0.02±0.01	0.74±0.07	90.3±2.37
03	72	1.3±0.11	0.04±0.3	1.12±0.21	84.33±2.84
04	96	1.84±0.3	0.04±0.3	1.37±0.05	92±1.15
05	120	2.34±0.5	0.06±0.03	1.50±0.9	91.64±6.36
06	144	2.24±0.08	0.04±0.02	1.75±0.5	87.01±1.21
07	168	2.19±0.04	0.04±0.03	1.78±0.03	82.31±3.28
08	192	2.17±0.05	0.04±0.12	1.72±0.4	88.33±0.81

Values are mean ± S.E.M (n=3)

Graph 4: Weight of biomass, extractable chitosan, and chitin from *Rhizopus oryzae* NCIM 879 with incubation time.



A. niger NCIM 545 had the highest growth rate with a maximal mycelial dry weight of 2.47gm with 0.06 g of chitosan after 8 d of cultivation, while *M.hiemalis* NCIM 873 and *R. oryzae* NCIM 879 grew

equally with a maximal biomass of 2.37g with 0.07g of chitosan and 2.34gm with 0.06g of chitosan after 6d and 8d of cultivation respectively.

The dry weight of mycelia (biomass) and extractable chitosan of the three genus increased with time. The fungal biomass increased rapidly during the first 74 hours of incubation and continued to increase until 120 hours after which the growth slowed down and the fungus appeared to enter the stationary phase.

The decline of the extractable chitosan seen in the time-culture curve might be due to physiological changes in the fungal cell wall (McGahren *et al.,* 1984). Chitosan is produced in the fungal cell wall by deacetylating its precursor, nascent chitin. During the exponential phase, the amount of extractable chitosan is relatively high, due to the active growth. Once the culture enters the stationary growth phase, more of the chitosan is anchored to the cell wall of the fungi by binding to chitin and other polysaccharides and extraction becomes more difficult. Therefore, although the fungal biomass was highest during the stationary growth phase, less chitosan is obtained. The results in graph 2, 3, 4 indicate that the late exponential growth phase of the fungus would give the best yield for chitosan.

Therefore, all fungi should be harvested at their late exponential growth phase and the content of extractable chitosan was determined. This is because different fungi have different growth rates, and the time needed for them to reach their late exponential growth phase will also different. If mycelia were harvested at a fixed incubation time, the amount of extractable chitosan might not be maximum. In light of the above results, the late exponential growth phase was determined for all genera of fungi used in this study. The late exponential phase of *Mucor hiemalis* was at 96 hour, and that of *Aspergillus niger, Rhizopus oryzae were* at 120 hours.

6.3. Medium optimization with relation to chitosan production

The maximal extractable chitosan content was determined for different carbon and nitrogen sources by harvesting at their late exponential phase and the results presented in Table no 6 and 7.

Table no 6: Comparison of yield of chitosan for different carbon sources

Strains	Carbon source				Nitrogen source	Mineral salts					Yield	
	Dextrose	Maltose	Sucrose	C.S	Peptone	K_2HPO_4	$MgSo_4$	$(NH4)_2SO_4$	NaCl	$CaCl_2$	Chitosan	Chitin
A.niger	X				X	X	X	X	X	X	0.03	1.06
		X			X	X	X	X	X	X	0.01	0.77
			X		X	X	X	X	X	X	0.05	1.58
				X	X	X	X	X	X	X	0.03	1.15
M.hiemalis	X				X	X	X	X	X	X	0.04	1.32
		X			X	X	X	X	X	X	0.01	1.08
			X		X	X	X	X	X	X	0.05	1.27
				X	X	X	X	X	X	X	0.03	1.10
R.oryzae	X				X	X	X	X	X	X	0.06	1.42
		X			X	X	X	X	X	X	0.04	1.27
			X		X	X	X	X	X	X	0.01	0.67
				X	X	X	X	X	X	X	0.04	1.27

Graph no 5. Comparison of yield of chitosan for different carbon sources

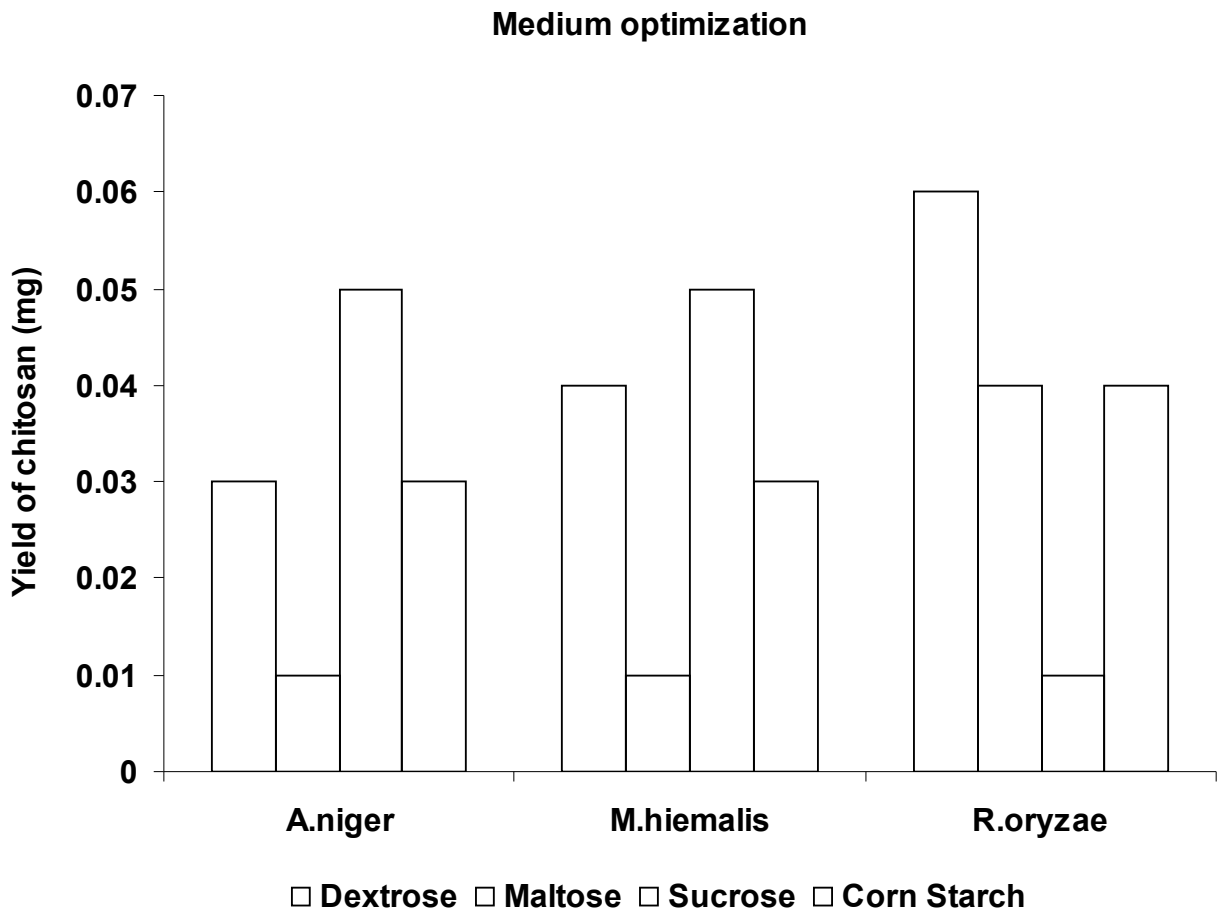
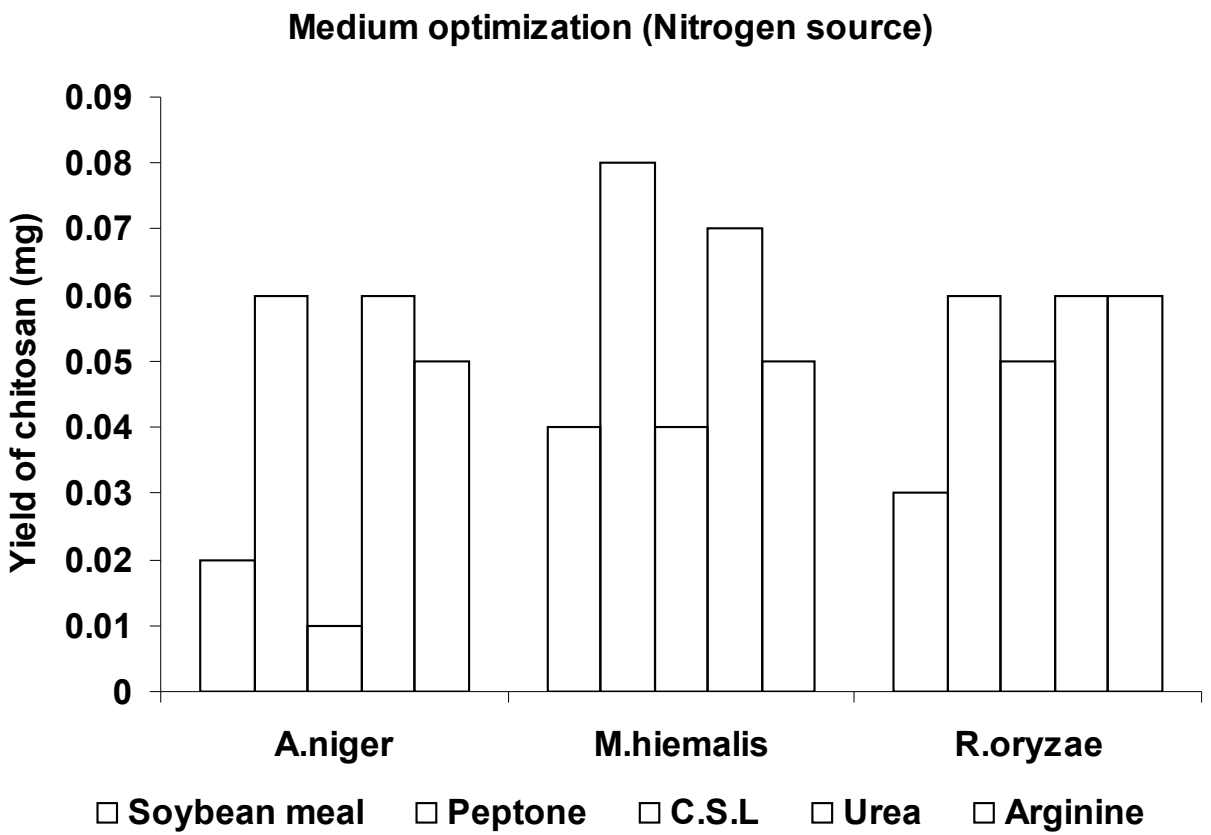


Table no 7: Comparison of yield of chitosan for different nitrogen sources

Strains	Nitrogen source					Carbon source	Mineral salts					Yield	
	SBM	Peptone	C.S.L	Urea	Arginine	Glucose	K_2HPO_4	$MgSo_4$	$(NH4)_2SO_4$	NaCl	$CaCl_2$	Chitosan	Chitin
A.niger	X					X	X	X	X	X	X	0.02	1.36
		X				X	X	X	X	X	X	0.06	1.27
			X			X	X	X	X	X	X	0.01	1.02
				X		X	X	X	X	X	X	0.06	1.58
					X	X	X	X	X	X	X	0.05	1.44
M.hiemalis	X					X	X	X	X	X	X	0.04	0.97
		X				X	X	X	X	X	X	0.08	1.14
			X			X	X	X	X	X	X	0.04	1.45
				X		X	X	X	X	X	X	0.07	1.41
					X	X	X	X	X	X	X	0.05	1.70
R.oryzae	X					X	X	X	X	X	X	0.03	1.12
		X				X	X	X	X	X	X	0.05	1.37
			X			X	X	X	X	X	X	0.05	1.15
				X		X	X	X	X	X	X	0.06	1.57
					X	X	X	X	X	X	X	0.06	1.63

Graph no 6: Comparison of yield of chitosan for different nitrogen sources



The inclusion of sucrose and glucose as a carbon source and urea as a nitrogen source led to the highest yield of chitosan and chitin in *Aspergillus niger.*

The inclusion of glucose and urea led to the highest yield of chitosan and chitin in *Mucor hiemalis*.

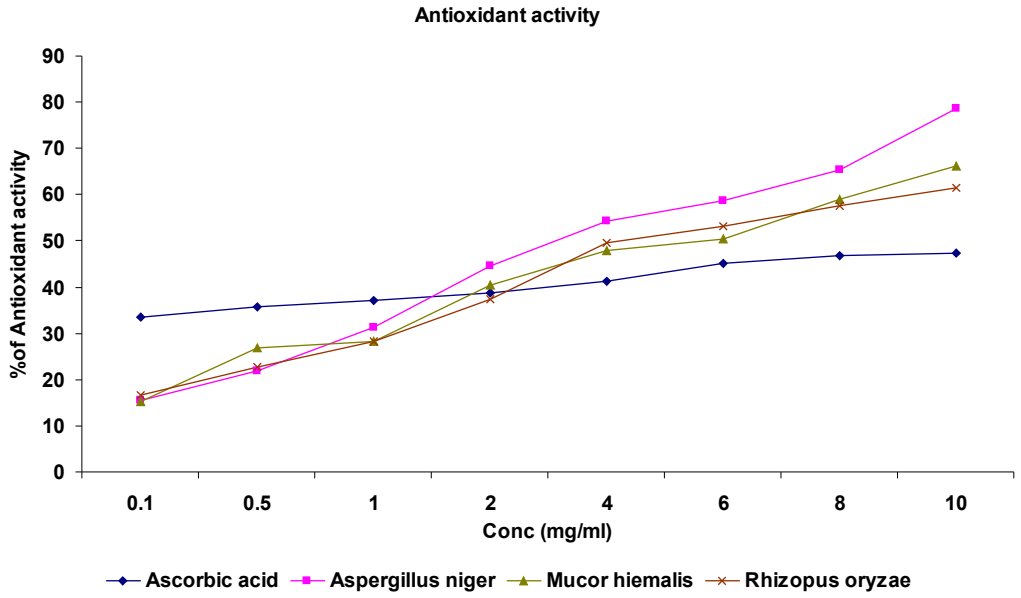
The inclusion of dextrose as a carbon source and urea as a nitrogen source led to the highest yield of chitosan and chitin in *Rhizopus oryzae.*

The results confirmed that the chitin and chitosan content of the fungi depends on the fungal strains, mycelial age and the composition of the growth medium. (Chatterjee S., et al., 2005)

Table no 8: Antioxidant activity of isolated chitosan

S.No	Conc of sample (mg/ml)	Percentage of Antioxidant activity			
		Ascorbic acid	*Aspergillus niger*	*Mucor hiemalis*	*Rhizopus oryzae*
01	0.1	33.42±0.14	15.45 ± 0.03	15.3±1.08	16.50±0.02
02	0.5	35.82±1.10	21.88 ± 0.1	26.79±0.22	22.66±0.78
03	1.0	37.14±0.15	31.43 ±0.03	28.34±0.23	28.36±0.27
04	2	38.89±0.21	44.70 ±0.16	40.4±0.20	37.30±0.58
05	4	41.14±0.09	54.33 ±0.07	47.99±0.45	49.55±0.38
06	6	45.01±0.11	58.57 ±0.23	50.41±0.83	53.06±0.67
07	8	46.78±0.20	65.42 ±0.26	59.07±0.39	57.52±0.12
08	10	47.44±0.13	78.52 ± 0.03	66.14±0.28	61.55±0.47
EC_{50} value	-	-	3.1mg/ml	5.3mg/ml	4.1mg/ml

Graph no 7: Antioxidant activity of isolated fungal chitosan and ascorbic acid



6.4. Antioxidant activity

The results of antioxidant activity shown in (Table no. 10) the isolated chitosan possess good antioxidant properties in scavenging ability on hydroxyl radicals. The EC_{50} value of the isolated chitosan was in the range of 3.1-5.3.

All the three isolated fungal chitosan shown good scavenging ability on DPPH free radical it may be due to the higher degree of deacetylation.

6.5. Characterization of chitosan by viscometry, FT-IR and ^{1}HNMR spectroscopy

6.5.1. Viscometry

Table no 9: Viscosity of isolated and commercial chitosan

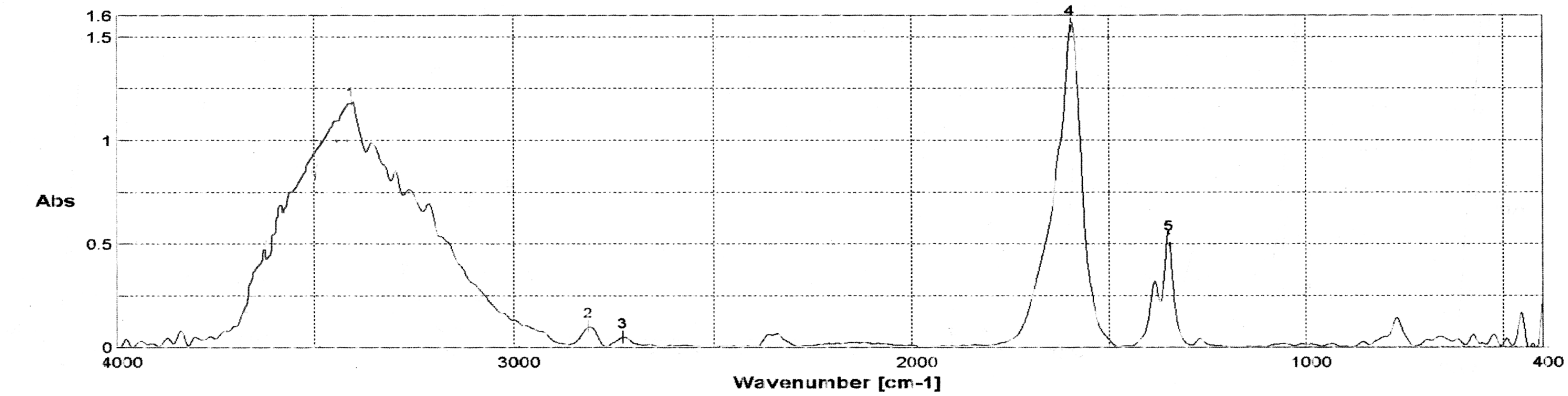
S.No	Samples	Viscosity (cP)
01	Commercial chitosan	123
02	*Aspergillus niger*	13.8
03	*Mucor hiemalis*	15.94
04	*Rhizopus oryzae*	17.8

The viscosity of the isolated fungal chitosan was in the range of 13-18 cP where as commercial chitosan having the viscosity of 123 cP.The viscosity of fungal chitosan was considerably lower than commercial chitosan highly viscous solutions are not desirable for industrial handling. A low viscosity chitosan from fungal mycelia as obtained in this work may facilitate easy handling in industries.

6.5.2. FT- IR spectroscopy

To prove that the acid extractable material contains chitosan, its FT-IR spectra were measured in comparison with IR spectrum of commercial chitosan from Sigma. All the three isolated chitosan shown similar FT- IR spectrum to that of the commercial chitosan. The result indicated that acid extractable material contains chitosan. The degree of

deacetylation was also calculated. The degree of deacetylation value of the fungal isolated chitosan was in the range of 80-99 % but the commercial chitosan shows only 64.96%.



Accumulation	16	Resolution	4 cm-1
Zero Filling	ON	Apodization	Cosine
Gain	8	Scanning Speed	2 mm/sec
Date/Time	10/22/2007 11:19AM	Update	10/22/2007 11:53AM
Operator	C.Geetha		
File Name	Standard		
Sample Name	Commercial chitosan		
Comment			

No.	cm-1	Abs	No.	cm-1	Abs	No.	cm-1	Abs	No.	cm-1	Abs	No.	cm-1	Abs
1	3408.57	1.16225	2	2813.63	0.0975037	3	2723.96	0.0483817	4	1595.31	1.56037	5	1345.11	0.520149

Fig 17; IR spectrum of commercial chitosan from Sigma chemicals

The degree of deacetylation was calculated using the following formula

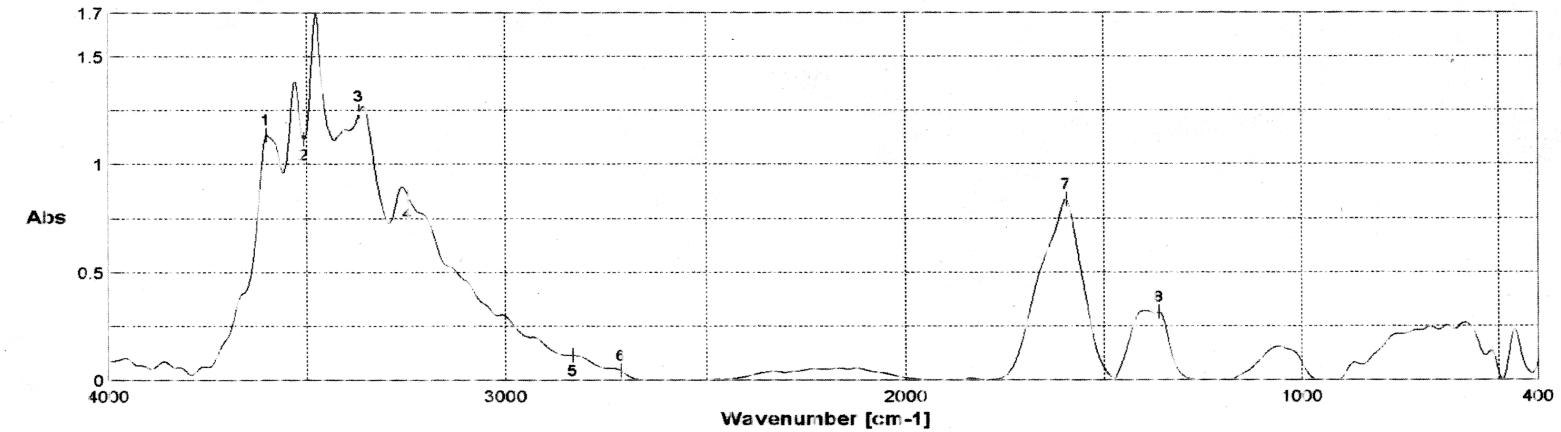
DDA = 118.883-[40.1647(A1655/A3450)]

= 118.883-[40.1647(1.56037/1.16225)]

= 118.883-[(40.1647 X 1.34254)]

=118.883-53.92

=64.96%



Accumulation	16	Resolution	4 cm-1
Zero Filling	ON	Apodization	Cosine
Gain	16	Aperture	7.077 mm
Scanning Speed	2 mm/sec		
Date/Time	8/28/2007 0:08PM	Update	8/28/2007 0:14PM
Operator	C.Geetha		
File Name	Memory#9		
Sample Name	A.nig		
Comment			

No.	cm-1	Abs	No.	cm-1	Abs	No.	cm-1	Abs	No.	cm-1	Abs	No.	cm-1	Abs
1	3603.34	1.13423	2	3506.92	1.11471	3	3370	1.24381	4	3247.64	0.845353	5	2936.77	0.112235
6	2713.35	0.0408639	7	1590.99	0.834236	8	1358.6	0.311357						

Fig 18. IR spectrum of isolated chitosan from *Aspergillus niger* NCIM 545

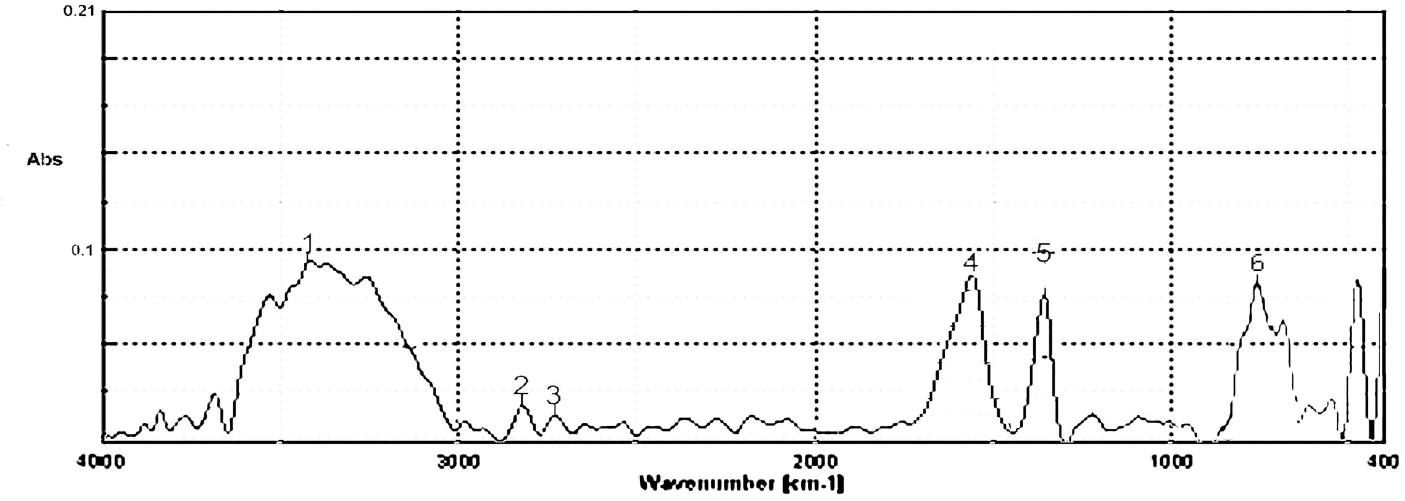
The degree of deacetylation was calculated using the following formula

DDA = 118.883-[40.1647-(A1655/A3450)]

=118.88-[40.1647-(0.834236/1.11471)]

=118.883-30.058796

=89%



Accumulation	16	Resolution	4 cm-1
Zero Filling	ON	Apodization	Cosine
Gain	4	Scanning Speed	2 mm/sec
Date/Time	11/14/2007 0:09PM	Update	11/14/2007 0:12PM
Operator	C.Geetha		
File Name	Samples		
Sample Name	Mucor hiemalis		
Comment			

No	cm-1	Abs	No.	cm-1	Abs	No.	cm-1	Abs	No.	cm-1	Abs	No.	cm-1	Abs
1	3455.81	0.081409	2	2921.63	0.0460146	3	2854.13	0.0257833	4	1555.31	0.0401456	5	1452.14	0.0624047
6	1398.14	0.069728												

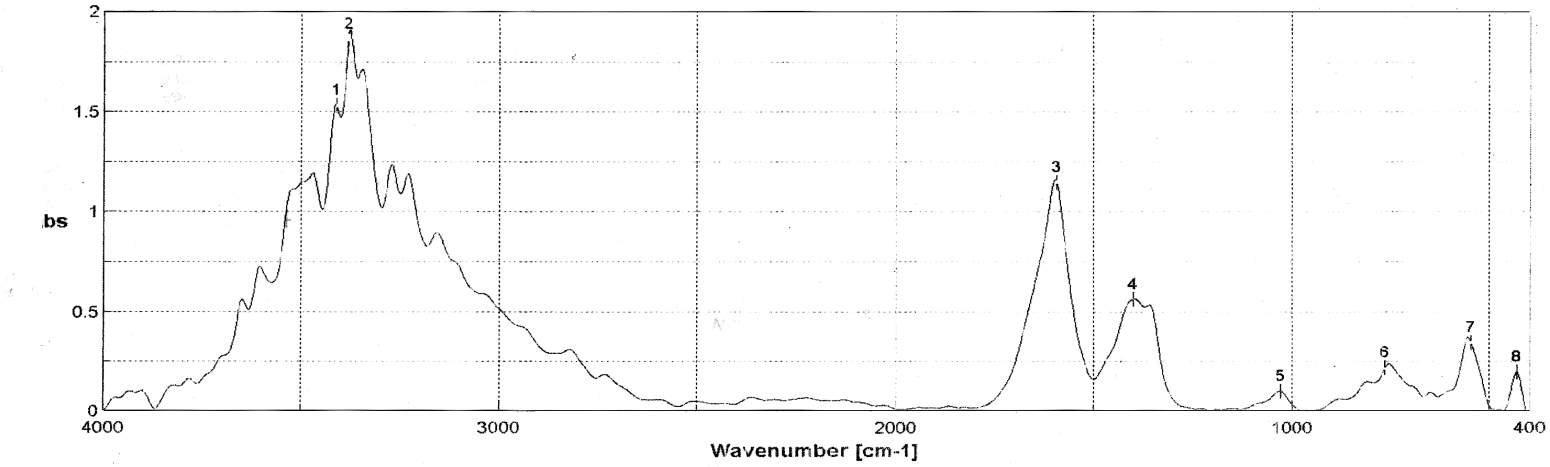
Fig 19. IR spectrum of isolated chitosan from *Mucor hiemalis* NCIM 873

DDA = 118.883-[40.1647(A1655/A3450)]

=118.883-[40.1647(0.0624047/0.081409)]

= 118.883-[(40.1647 X 0.4937347)]

=99.07%



Accumulation	16	Resolution	4 cm-1
Zero Filling	ON	Apodization	Cosine
Gain	32	Scanning Speed	2 mm/sec
Date/Time	11/20/2007 3:03PM	Update	12/17/2007 11:17AM
Operator	C.Geetha		
File Name	Samples		
Sample Name	Rhizopus oryzae		
Comment			

No.	cm-1	Abs	No.	cm-1	Abs	No.	cm-1	Abs	No.	cm-1	Abs	No.	cm-1	Abs
1	3411.46	1.53202	2	3380.6	1.86368	3	1593.88	0.823136	4	1398.14	0.563303	5	1027.87	0.100946
6	765.601	0.218771	7	546.72	0.344796	8	431.012	0.197534						

Fig 20. IR spectrum of isolated chitosan from *Rhizopus oryzae* NCIM 879

DDA = 118.883-[40.1647(A1655/A3450)]
=118.883-[40.1647(0.823136/1.53202)]
= 118.883[(40.1647 X 0.537288)]
=118.883-21.5800
=97.30%

7.6.3. ^{1}H NMR spectroscopoy

The ^{1}H NMR spectra was measured in comparison to commercial chitosan and it proved that the isolated fraction contains chitosan. The isolated fraction showed less intense peak at 2.0 ppm (acetyl group) as compared to commercial chitosan confirming that the isolated chitosan had a higher degree of deacetylation.

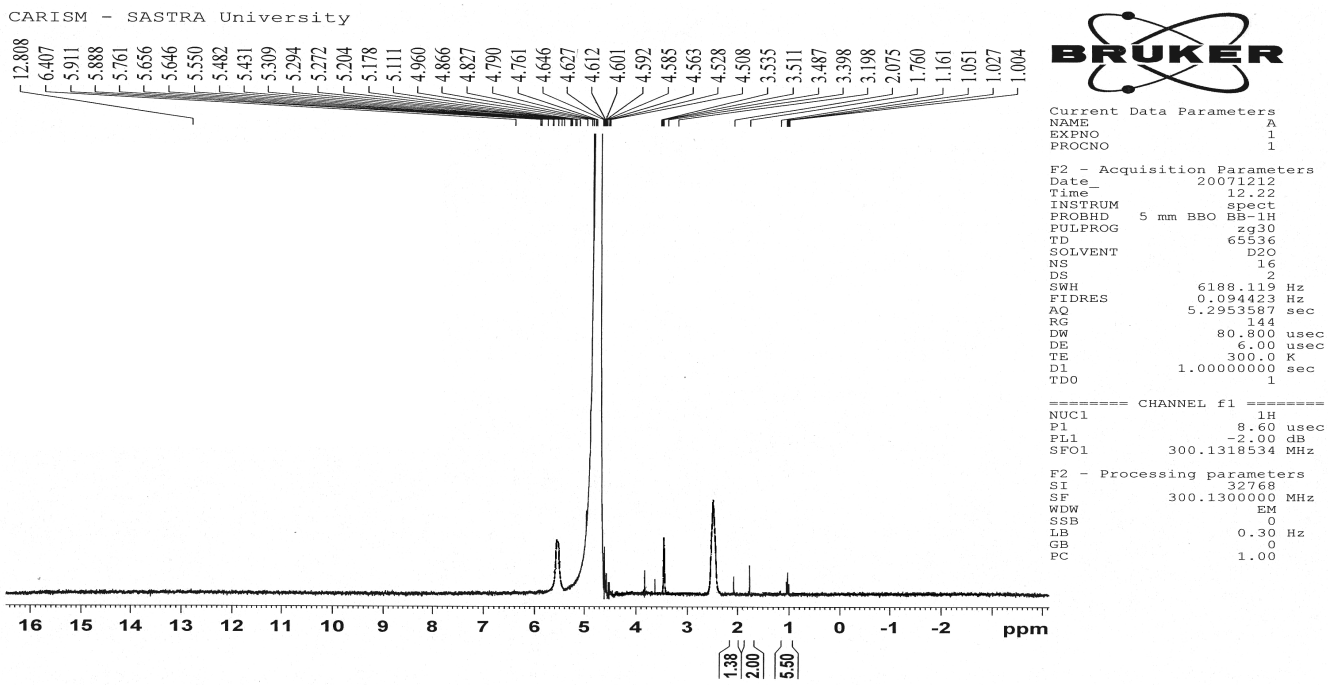


Fig 21. ^{1}HNMR spectrum of commercial chitosan from Sigma chemicals

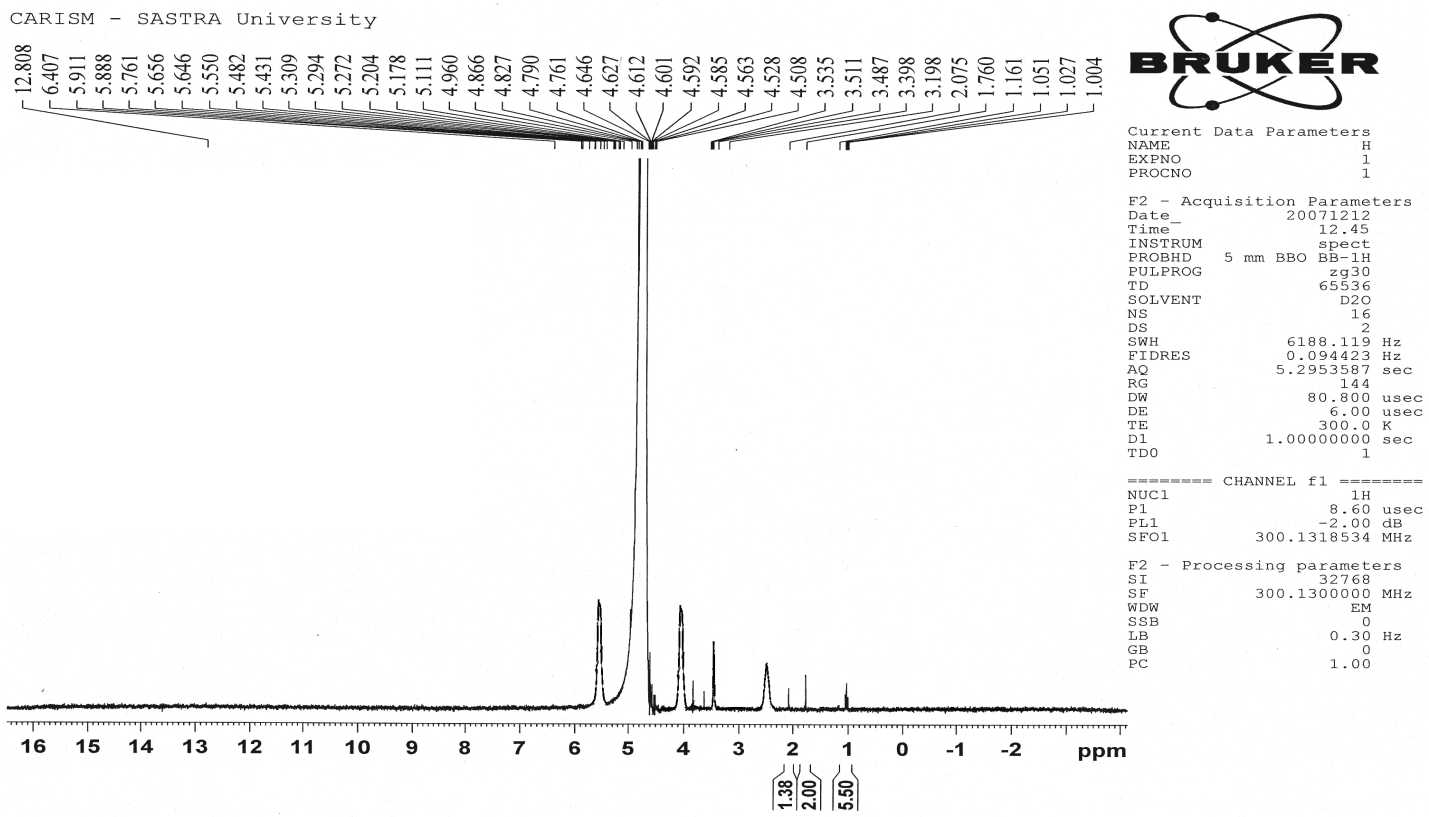


Fig 22.[1]HNMR spectrum of isolated chitosan from *Aspergillus niger* NCIM 545

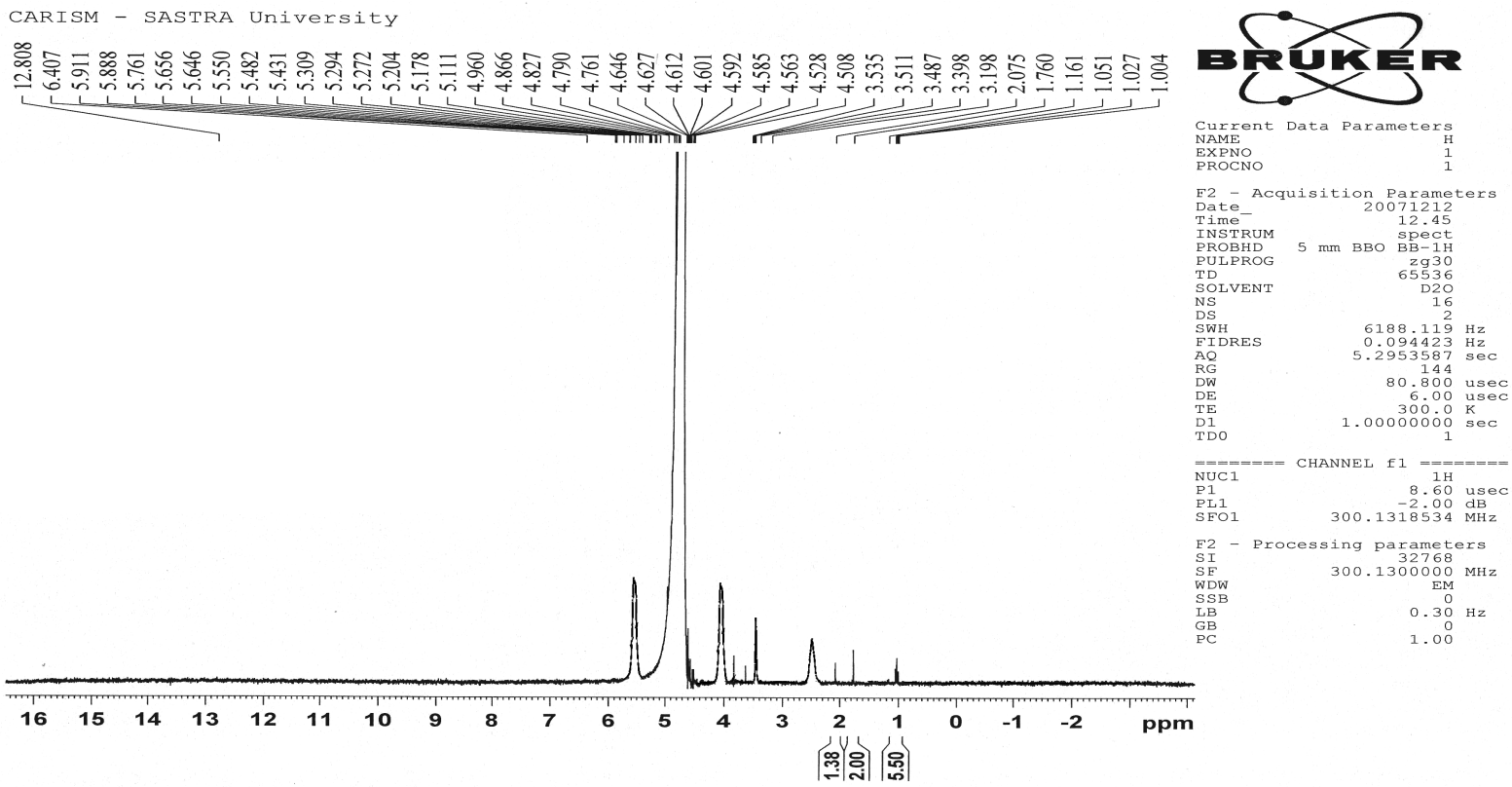


Fig 23.[1]HNMR spectrum of isolated chitosan from *Mucor hiemalis* NCIM 873

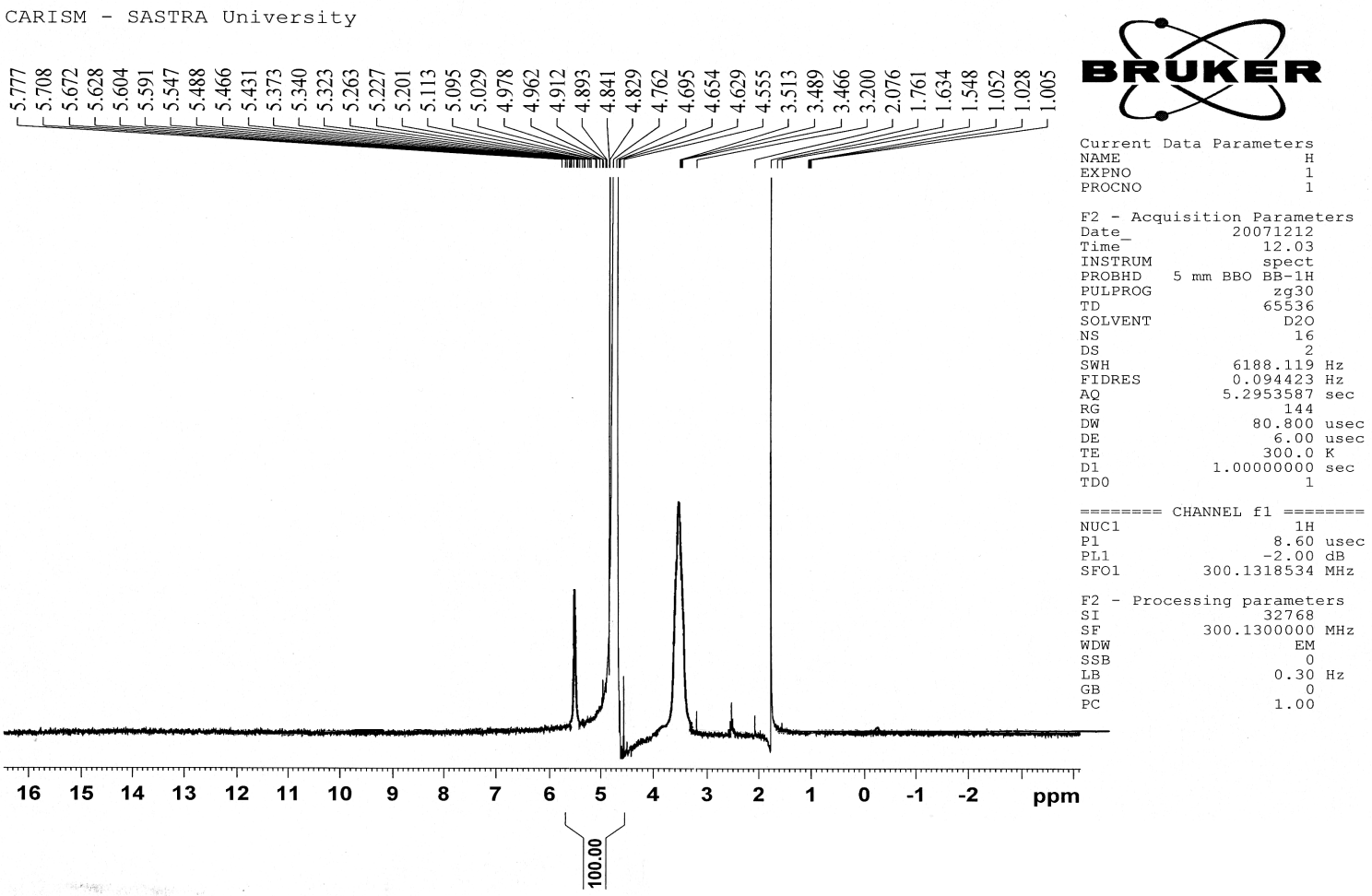


Fig. 24:[1]HNMR spectrum of isolated chitosan from *Rhizopus oryzae* NCIM 879

The degree of deacetylation of the isolated chitosan was 80-99%it is relatively higher than that of the commercial chitosan. The degree of deacetylation is an important parameter affecting the physicochemical properties of chitosan. Chitosan with high degree of deacetylation has high positive charges and is more suitable for food applications as a coagulating or chelating agent, a clarifying agent or an antimicrobial agent.

6.7. ANTIMICROBIAL STUDIES

6.7.1. Antibacterial studies

The isolated fungal chitosan and commercial chitosan were tested for their anti bacterial activity against Gram positive and Gram negative organisms by disc diffusion method using Ciprofloxacin (5µg/disc) as standard.

Gram Positive Organisms

Both *Staphylococcus aureus* and *Bacillus subtilis was* found to be sensitive to all the isolated fungal chitosan and commercial chitosan. The chitosan did not show sensitivity against *Micrococcus luteus* except chitosan isolated from *M.hiemalis.*

Gram Negative Organisms

Both *E.coli* and clinical isolate of *E.coli* was found to be highly sensitive to all the isolated fungal chitosan and commercial chitosan. *Pseudomonas aeruginosa* was found to be moderately sensitive towards all the isolated chitosan and commercial chitosan.

The growth inhibitory effect of the isolated chitosan against bacteria is due to free-NH_3^+ groups, which are responsible for the binding of negative charges on the bacterial cell surface to bring about antibacterial activity (Chen C., et al., 1998).

6.7.2. Anti Fungal Activity

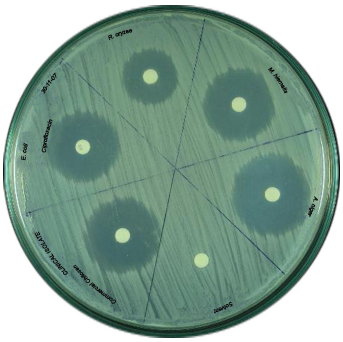
All the isolated fungal chitosan and commercial chitosan were tested for their activity against *Candida albicans* and *Aspergillus niger* by agar diffusion method using Fluconazole (10 μg/disc) as the standard.All the isolated fungal chitosan and commercial chitosan were found to be inactive against both *Aspergillus niger* and *Candida albicans*.

As per earlier reports, the fungal chitosan showed good antifungal activity to certain genus and the derivatives of the chitosan showed better growth inhibitory activity than compared to normal chitosan. (Liu et al., 2004)

Table no 10: Antibacterial l activity of isolated and commercial chitosan

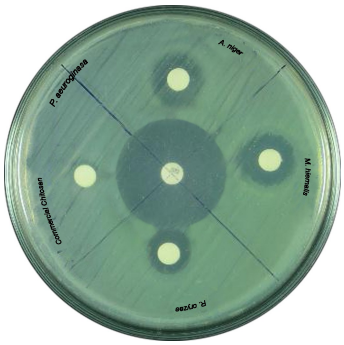
Samples tested	Diameter of Zone of inhibition (mm) (n=2)					
	Gram negative bacteria			Gram positive bacteria		
	E.Coli	**Clinical isolate *(E.coli)***	***P.aeruginosa***	***S.aureus***	***B.subtilis***	***M.luteus***
Commercial chitosan	30±0.5	30±0.2	10.33±0.3	16.33±0.7	27	-
A.niger	31±0.5	33±0.5	15.32±0.6	19±0.4	27.67±0.2	12.32±0.5
M.hiemalis	34.66±0.3	28.33±0.3	14.66±0.33	21.67±0.2	21.33±0.3	-
R.oryzae	33.33±0.3	22±0.5	13.3±0.6	19	20.33±0.3	-
Ciprofloxacin	37	34	39	35	30	31

Fig. 25 : Anti bacterial activity of isolated and commercial chitosan on Muller Hinton Agar by disc diffusion method.

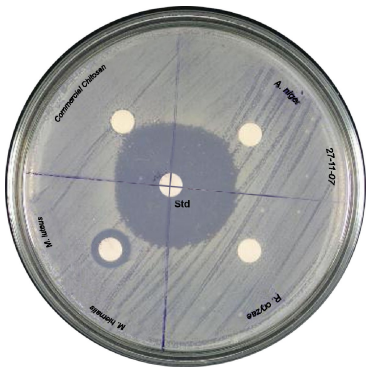


Clinical isolate of *E.coli*

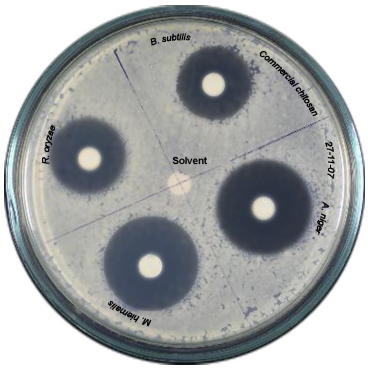
E.coli



P.aeruginosa



M.luteus



B.subtilis

S.aureus

VII. SUMMARY AND CONCLUSION

As the first step towards the production of high quality chitosan biopolymer with maximum industrial, pharmaceutical and medical application we could identify three species (*Aspergillus niger NCIM 545, Mucor hiemalis NCIM 873* were used as test organisms and *Rhizopus oryzae NCIM* 879 was used as a reference strain for the production of chitosan biopolymer) with the desired characteristics.

The chitosan produced using the above three species was comparable to the commercial chitosan and in some properties was even superior to the commercial source like the present finding typically has at least 80-99% of deacetylation. This level of deacetylation provides high quality chitosan with consistent properties that is readily soluble in a slightly acidic solution.

Among the three species *Mucor hiemalis* NCIM 873 was found to give a maximal yield of chitosan compared with other two genus.

The viscosity of the isolated fungal chitosan was in the range of 13-18 cP where as commercial chitosan having the viscosity of 123 cP (Table no 8) the results was correlated as described (Pochanavanich and Suntornsuk. 2002)

The profile of both isolated and commercial chitosan showed similar FT-IR and NMR spectra.

The isolated chitosan was found to possess good antioxidant properties (scavenging ability on DPPH free radicals) having the EC_{50} value in the range of 3.1-5.3.

All the three isolated fungal chitosan showed good antibacterial activity against Gram positive bacteria and Gram negative bacteria,

including pathogenic *E.coli.*

Based on the results it may be concluded that the mycelium of *Mucor hiemalis, Rhizopus oryzae and A.niger* could be used as good alternative sources for commercial chitosan production. The achievement of desired physicochemical properties such as higher degree of deacetylation and lower viscosity of the isolated fungal chitosan than the commercial chitosan indicates the superiority for using it for various pharmaceutical and medicinal applications. Fungal chitosan with alleged antioxidant properties may be used as a source of antioxidant in the pharmaceutical industry. The antibacterial activity of fungal chitosan against, *S.aureus*, *E.coli including* a and clinical isolate of *E.coli* revealed that it can be used for therapeutic purposes.

Further extension on the work could involve study on the use of cheaper substrates and mycelial wastes of both antibiotic and citric acid industry in the production of chitosan. Studies on the effects of promoters and inhibitors on the production of chitosan [eg; D-Psicose (Yoshihara K., et al., 2003) Gibberellic acid (Chatterjee S., et al., 2007)] could also be done. The genetic manipulation of the fungal system in the near future may also increase the production of this important biopolymer **CHITOSAN.**

BIBLIOGRAPHY

Alexander Steinbuchel. Perspectives for biotechnological production and utilization of biopolymers: metabolic engineering of polyhydroxy alkanoate biosynthesis pathway as a successful example. *Macromol.biosci*.2001; **1**: 1-24.

Aneja K.R.. Experiments in microbiology plant pathology and tissue culture. Second edition. New Delhi, V.S.Johri Publisher.1996. p 52.

Arcidiacono S, Kaplan D.L.. Molecular weight distribution of chitosan isolated from *Mucor rouxii* under different culture and processing conditions. *Biotechnol Bioeng* 1992;**39**:281–286.

Arikan S, Lozano-Chiu M, Paetznick V, Rex J.H.. *In vitro* susceptibility testing methods for caspofungin against *Aspergillus* and *Fusarium* isolates. *Antimicrob. Agents Chemother*. 2001;**45**:327-330.

Arikan S, Rex J.H.. New agents for treatment of systemic fungal infections. *Emerging Drugs* 2000;**5**:135-160.

Asada Y, Miyake J, Miyake M, Kurane R, Tokiwa Y.. Perspectives for biotechnological production of and utilization of biopolymer.*Int. j. Biol. Macromol* 1999; **25**: 37.

Atlas R.M, Perks L.G, Brown A.E.. Laboratory Manual of Experimental Microbiology, Mosby Publishers, Louisville, KY: 1995.

Baxter A, Taylor D.M, Roberts G.A. F.. Improved method for infra red determination of the degree of N-acetylation of chitosan. *Int J Biol Macromol* 1992; **14**: 166-169.

Brugnerotto J, Lizardi J, Goycoolea F.M, Argiielles M.W, Desbrieres J, Rinaudo M.. An infrared investigation in relation with chitin and chitosan characterization. *Polymer* 2001; **42**: 2580–3569.

Carlile M.J, Watkinson S.C, Gooday G.W.. The Fungi. Second edition. London Academic press;2001.

Cappuccino JG, Sherman N.. Microbiology – A Laboratory Manual, 3rd edition. New York: Benjamin Cummings Publishing Company; 1992. 51-66.

Chan H.Y, Chen M.H, Chuang S.H, Chang H.O, Wang S.H, Chen J.C, et al., (2005) U.S. patent 6,890,91 B2.

Chatterjee S, Chatterjee S, Chatterjee B.P, Guha A.K.. Enhancement of growth and chitosan production by *Rhizopus oryzae* in whey medium by plant growth hormones. *Int J Biol Macromol*. 2007;**12**: [Epub ahead of print].

Chatterjee S., Adhya M., Guha A.K., Chatterjee B.P.. Chitosan from *Mucor rouxii*: production and physico-chemical characterization. *Process Biochemistry* 2005 ;**40** : 395–400

Chen Y.M, Chung Y.C, Wang L.W, Chen K.T, Li S.Y.. Antibacterial properties of chitosan in waterborne pathogen. *J Environ Sci Health A Tox Hazard Subst Environ Eng* 2002;**37**(7):1379-90.

Chen C, Lian W, Isai G.. Antimicrobial effects of N-sulfonated and N-sulfobenzyl chitosan and applications in preservation. *J. Food Prot* 1998;**61**:1124–1128.

Chen M.H, Chan H.Y, Su-Hui chuang.. (2001)U.S. patent 6,255,085 B1.

Chen M.H, Chan H.Y, Su-Hui chuang.. (2002)U.S. patent 6,485,946 B1.

Chen M.H, Chan H.Y, Su-Hui chuang..(2002)U.S. patent 6,399,338 B1.

Davidson G.R, Konig A, Brian Ripley, Macglip N.A.. (2003) U.S. patent 6,638,918 B2

De Hoog G.S, Guarro J, Gene J, Figueras M.J. Atlas of Clinical Fungi. 2nd ed. vol.1. Centraalbureau voor Schimmelcultures. Utrecht, The Netherlands; 2000.

Di Mario F, Rapana P, Tomati U, Galli E. Chitin and chitosan from Basidiomycetes. *Int J Biol Macromol* 2007 [Epub ahead of print].

Domsch K.H, Gams W, Anderson T.. Compendium of soil fungi, Academic

Press, London, United Kingdom. 1980. 703-709.

Domszy G.K, Roberts G.A.F. Muzzarelli, R.A.A., Pariser, E.R., (eds), Proceedings of the first International Conference on chitin/chitosan. *MIT Sea Grant Report* 1985. 421.

Frater J.L, Hall G.S, Procop G.W.. Histologic features of zygomycosis - Emphasis on perineural invasion and fungal morphology. *Arch Pathol Lab Med* 2001;**125**:375-378.

Helander I, Latva-Kala M, Lounatmaa K. Permeabilizing action of polyethyleneimine on *Salmonella typhimurium* involves disruption of the outer membrane and interactions with lipopolysaccharide. *Microbiology* 2003; **344**:385–390.

Jeon Y.J, Park P.J, Kim S.K.. Antimicrobial effect of chito-oligosaccharides produced by bioreactor. *Carbohydr. Polym* 2001;**44**: 71–76.

Jin Woo Lee, Yeomans W.G, Allen A.L, Fang Deng, Gross R.A, Kaplan D.l.. (2003) U.S. patent 6,534,294 B1.

Kobayashi M, Watanabe T, Suzuki S, Suzuki M.. Effect of N-acetylchitohexaose against *Candida albicans* infection of tumor bearing mice. *Microbiology and Immunology* 1990; **34**: 413–426.

Kim T.H, Jiang H.L, Jere D, Park I.K, Cho M.H, Nah J.W.. Chemical modification of chitosan as a gene carrier *in vitro* and *in vivo*. *Prog. Polym. Sci*. 2007; **32:**726–753.

Lavertu M, Xia Z, Serreqi A.N , Berrada M, Rodrigues A, Wang D, Buschmann M.D., Gupta A. A.. validated ^{1}H NMR method for the determination of the degree of deacetylation of chitosan. *J. Pharm. Biomed. Anal* 2003; 32: 1149, 1158.

Lin H.Y, Chou C.C.. Antioxidant activities of water-soluble disaccharide chitosan derivatives. *Food Research International* 2004; **37**:883–889.

Liu H, Du Y, Wang X, Sun L.. Chitosan kills through cell membrane

damage. *International journal of food microbiology* 2004;**95**(2):147-155.

Mackie, McCartney. Practical Medical Microbiology, 14th ed., Churchill Livingstone, New York: 1996. 95-130.

Madigan M.T, Martinko J.M, Parker J.. Biology of microorganisms. Ninth edition, prentice-Hall, Upper Saddle Riverzoo.2001.

McGahren W.J, Perkinson G.A, Growich J.A, Leese R.A, Ellestad G.A.. Chitosan by fermentation. *Process Biochem* 1984; **19**: 88-90.

Mensor L.L, Menezes F.S, Leitao G.G, Reis A.S, Dos santos T.C, Coube C.S, Leitao S.G.. Screening of Brazilian plant extracts for Antioxidant activity by the use of DPPH free radical method. *Phytother. Res* 2001;**15**:127-130.

Muzzarelli R.A.A, Rochetti R, Stanic V, Weckx M, Methods for the determination of the degree of acetylation of chitin and chitosan. Chitin hand book. European Chitin Society 1997: 109–19.

My lien Dao. (2007) U.S. patent 7,157,242 B1.

Nakashima T, Matsuo M, Bin Y, Nakano Y, Komemushi S, Sakagami Y.. Mechanical properties and antibacterial efficacy of chitosan films. *Biocontrol Sci* 2006 ; **11**(1):27-36.

New N, Stevens W.F.. Effect of urea on fungal chitosan production in solid substrate fermentation. *Process Biochemistry* 2004; **39**: 1639–1642.

Niederhofer A, Muller B.W.. A method for direct preparation of chitosan with low molecular weight from fungi. *European Journal of Pharmaceutics and Biopharmaceutics* 2004; **57:**101–105.

Nwe N, Stevens WF, Montet D, Tokura S, Tamura H.. Decomposition of myceliar matrix and extraction of chitosan from *Gongronella butleri* USDB 0201 and *Absidia coerulea* ATCC 14076. *Int J Biol Macromol* 2007; [Epub ahead of print].

Okmi Park, Jun Watanabe, Tohru Chiba, Isao Endo. .(1993) U.S. patent

5,232,842.

Patino J.F, Castro D, Valencia A, Morales P.. Necrotizing soft tissue lesions after a volcanic cataclysm. World J. Surg 1991;15:240-247.

Petersen K, Nielsen P.V, Bertelsen G, Lawther M, Olsen M.B, Nilson N.H.. et al., potential of biobased materials for food packaging. Trends in food science and technology 1999; 10(2):52-58.

Pochanavanich P, Suntornsuk W.. Fungal chitosan production and its characterization. *Lett. Appl. Microbiol* 2002; **35**: 17–21.

Podolski J.S, Martinez M.L.. (1999) U.S Patent 5,965,144

Premraj R, Doble M.. Biodegradation of polymers. *Indian journal of biotechnology* 2005;**4**:186-193.

Qin C, Du Y, Xiao L, Li Z, Gao X. Enzymic preparation of water-soluble chitosan and their anti tumor activity. *Int. J. Biol. Macromol* 2003;**31**: 111–17.

Rabea E.I, Badawy M, Rogge T.M, Stevens C.V, Steurbaut W, Hofte M, et al, Enhancement of fungicidal and insecticidal activity by reductive alkylation of chitosan. Pest Manag Sci 2006 ; 62(9):890-97.

Roller S, Covill N.. The antifungal properties of chitosan in laboratory media and apple juice. *Int J Food Microbiol* 1999; **47**:67–77.

Rout S.K., Priya W.. Determining degree of Deacetylation and purity of crawfish chitin and chitosan Using FT-IR Spectroscopy. *IFT;* Annual meeting June 23-27 2001..New Orleans, LA.

Sabnis S, Block L.H.. Improved infrared spectroscopic method for the analysis of degree of N-deacetylation of chitosan. *Polym Bull* 1997;**39**:67-71.

Schipper M.A. A revision of the genus *Rhizopus*. The *Rhizopus stolonifer* group and *Rhizopus oryzae*. Studies in Mycology 1984; 25:1-19.

Shahidi F, Arachchi J, Jeon Y.J.. Food applications of chitin and chitosan. *Trends in Food Science and Technology* 1999;**10:** 37–51.

Shimahara K, Takiguchi Y, Kobayashi T, Uda K, Sannan T.. Screening of Mucoraceae strains suitable for chitosan production. In Chitin and Chitosan: Sources, chemistry, biochemistry, physical properties and applications. Elsevier; New York: 1988.171-178.

Sugar A.M, Liu X.P.. Combination antifungal therapy in treatment of murine pulmonary mucormycosis: Roles of quinolones and azoles. *Antimicrob. Agents Chemother* 2000;**44**:2004-2006.

Sutton D.A, Fothergill A.W, Rinaldi M.G.. Guide to Clinically Significant Fungi. 1st ed. Williams and Wilkins; Baltimore; 1998.

Tanverr A, Ahmad G, Khan D.. Reporting degree of deacetylation values of chitosan: the influence of analytical methods. *J pharm pharmaceut sci* 2002;**5**(3): 205—212.

Tharanathan R.N, Kittur F.S.. Chitin – the undisputed biomolecule of great potential. *Crit. Rev. Food Sci. Nutr*. 2003; **43**: 61–87.

Thayaza ,Tania , Perira, Benicio de Barros, Galba maria.. Growth of *cunninghamella elegans* UCP 542 and production of chitin and chitosan using yam bean medium. *Microbial biotechnology* 2007;**10** (1):242-249 .

Tianwei T, Binwu W, Xinyuan S.. Separation of chihtosan from *Penicillium chrysogenum* mycelium and its applications. *Journal of bioactive and compatible polymers* 2002;**17** (3): 173-182.

Vilai Rungsardthong, Nijarin Wongvuttanakul, Nilada Kongpien, Pachara Chotiwaranon. Application of fungal chitosan for clarification of apple juice. *Int J Biol Macromol.* 2005;**12**: [Epub ahead of print].

Weiyu Fan, Bohlman J.A, Trinkle J.R.. (2005) U.S. Patent 6,972,284 B2.

White S.A, Farina P.R.. Production and isolation of chitosan from *Mucor rouxii. Appl. Environ.Microbial* 1979;**38**: 323-328.

Wu T, Zivanovic S, Draughon F.A, Conway W.S, Sams C.E.. Physicochemical properties and bioactivity of fungal chitin and

chitosan. *J Agric Food Chem* 2005 18;**53**(10):3888-94.

Xiaohui Wang, Yumin Du, Hui Liu.. Preparation, characterization and antimicrobial activity of chitosan–Zn complex. *Carbohydrate Polymers* 2004; **56:**21–26.

Xie W, Xu P, Liu Q.. Antioxidant activity of water-soluble chitosan derivatives. *Bioorganic and Medicinal Chemistry Letters* 2001; **11**: 1699–1701.

Xing R, Yu H, Liu S, Zhang W, Zhang Q, Li Z, et al., Antioxidative activity of differently regioselective chitosan sulfates *in vitro*. Bioorganic and Medicinal Chemistry 2005;**13**: 1387–1392.

Yena M.T, Tsengb Y.H, Lia R.C, Maua J.L.. Antioxidant properties of fungal chitosan from shiitake stipes. *LWT* 2007; **40**: 255–261.

Yoshihara K, Shinohara Y, Hirotsu T, Izumori K.. Chitosan productivity enhancement in *Rhizopus oryzae* YPF-61A by D-psicose. *J Biosci Bioeng* 2003; **95**(3):293-297.

Zamani A, Edebo L, Sjostrom B, Taherzadeh M.J.. Extraction and precipitation of chitosan from cell wall of zygomycetes fungi by dilute sulfuric acid. *Biomacromolecules.* 2007 : [Epub ahead of print].

Zhou W, Yuan H, Wang J, Yao J.. Production, purification and characterization of chitosanase produced by *Gongronella* species. JG. *Lett Appl Microbiol* 2007 5: [Epub ahead of print].

http://www.plastictrends.net

http://www.vanderbilt.edu

www.en.wikipedia.org.wiki Antioxidant (Accessed 20thDec2007).

www.rice.edu/~jenky/html (Accessed 20thDec2007).